Article

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Liposomes versus Lipid Nanoparticles: Comparative Study of Lipid-Based Systems as Oryzalin Carriers for the Treatment of Leishmaniasis

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Main-stay in treatment of leishmaniasis relies on chemotherapy but none of the current drugs combines high activity and low toxicity at affordable costs. Dinitroanilines are a new class of drugs with proved *in vitro* antileishmanial activity. However the development of their pharmaceutical formulations has been compromised by low water solubility and low accumulation in diseased organs. These limitations can be overcome by incorporation in lipid-based nanoformulations such as liposomes and solid lipid nanoparticles. In previous work this strategy was already followed with the incorporation of a dinitroaniline, oryzalin, resulting in the improvement of the biodistribution profile. The present work aims at demonstrating the *in vitro* and *in vivo* therapeutic activity of these oryzalin nanoformulations, and establishing a systematic comparison of both systems. After oryzalin incorporation suitable physicochemical properties for parenteral administration were obtained. Nanoformulations revealed reduced cytotoxicity and haemolytic activity when compared with free-oryzalin, while retaining the *in vitro* intracellular activity. Therapeutic activity, assessed in a murine model of visceral leishmaniasis, was evaluated in terms of number of administrations, dose-response and influence of the lipid excipient. Results demonstrate the superiority of both oryzalin nanoformulations on the reduction of parasitic burden in liver and spleen as compared to the control group (84 to 91%) and similar to Glucantime®. A strong reduction in ED_{50} values (3 to 65 fold) as compared to free-oryzalin was also obtained, depending on the organ and nanoformulation used. Both oryzalin nanoformulations are potential candidates as therapeutic agents against visceral leishmaniasis.

KEYWORDS: *Visceral Leishmaniasis, Liposomes, Solid Lipid Nanoparticles, Dinitroanilines, Oryzalin, In Vitro Activity, In Vivo Activity.*

INTRODUCTION

Leishmaniasis, a neglected tropical disease caused by protozoa parasites of the genus *Leishmania*, is characterised by diverse and complex clinical manifestations ranging from skin lesions to serious disfigurement and fatal systemic infection.[1] The disease is caused by the infective promastigote form of the parasite that multiply as amastigotes within cells of the mononuclear phagocytic system (MPS), mainly macrophages. Despite infecting around 14 million people worldwide in 98 countries and with more than 350 million people at risk of infection, to date no effective vaccine is available and control strategies rely primarily on chemotherapy to alleviate the disease and on vector control to reduce transmission.[2] Chemotherapy treatments based on pentavalent antimonials have been the first-line choice for over 70 years in most parts of the world while second-line treatments include drugs like amphotericin B, paromomycin, miltefosine or liposomal amphotericin B. However, most of these treatment options are limited by several factors, such as variable efficacy, emergence of resistance, long duration, severe side effects and/or high costs.[3,4] For this reason it is of utmost importance to look for new drugs and drug targets for the treatment of leishmaniasis.

Microtubules are cellular structures formed from α and β tubulins constituting one of the major cytoskeletal components in eukaryotic cells, including *Leishmania* spp. As such they are among the most promising new drug targets for leishmaniasis chemotherapy.[5] Microtubule inhibitors, such the dinitroaniline family of herbicides, are currently tested for the treatment of this disease. This class of drugs

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has already proved to be active against several types of pathogenic protozoa, such as *Trypanosoma* spp.,[6] *Leishmania* spp.[7–9] and *Plasmodium falciparum*.[10] The potential of dinitroanilines as antiparasitic agents arises from their selectivity to protozoan parasites tubulin and lack of binding affinity to mammalian tubulin making these molecules safe to animals.[11] Their antileishmanial properties were first described by Chan et al.[11] who showed that one dinitroaniline, trifluralin (TFL), inhibited the proliferation and differentiation of *L. amazonensis*. It was also proved that another dinitroaniline, oryzalin (ORZ), is active *in vitro* against *Leishmania* strains responsible for visceral (VL)[7,9] and cutaneous leishmaniasis.[8] Despite this anticipated antileishmanial potential, the development of pharmaceutical formulations of active dinitroanilines for parenteral administration has been compromised due to its low water solubility[12] and low accumulation in the diseased organs.[13] These features pose a major challenge to their pharmaceutical formulation and clinical application.[14] The association of these agents with suitable nano drug delivery systems (nanoDDS) represents a strategy not only to overcome these problems, but also to improve the therapeutic index while reducing potential side effects of this drug.

One of the most extensively used lipid-based nanoDDS able to promote a selective and targeted delivery of various therapeutic agents are liposomes.[15] These phospholipidic vesicles are suitable carriers for antileishmanial drugs as they are naturally taken up by macrophages in the liver and in the spleen,[16] the main reservoirs of *Leishmania* parasites. The use of liposomal formulations resulted in a substantial increase of antileishmanial activity of several drugs as compared to the free drug.[17–20] Nevertheless, the potential advantages of using nanoDDS for current antileishmanial drugs may be limited by the pre-existence of resistance to those drugs (e.g., pentavalent antimonials or miltefosine) or by prohibitive high costs (liposomal amphotericin B).[21,22]

Two dinitroanilines, TFL and ORZ, are new therapeutic agents that present potential antileishmanial properties that could be enhanced by their association with nanoDDS. Carvalheiro et al.[23,24] have developed liposomal formulations of TFL that demonstrated a superior antileishmanial activity as compared to the free drug in a murine model of VL (*L. donovani*) and in the treatment of experimental canine leishmaniasis (*L. infantum*).[24] Furthermore Lopes et al.[25] reported the incorporation of ORZ in appropriate liposomal formulations. These new ORZ formulations were responsible for an increased ORZ solubility and an increased delivery of the drug to the main organs of leishmanial infection.[13] Nevertheless the *in vitro* and *in vivo* therapeutic activity of ORZ liposomes (Lip-ORZ) was not demonstrated, being one of the objectives of this work. Another objective was the clarification of possible advantages on using other type of lipid-based nanoDDS, solid lipid nanoparticles (LNP), instead of liposomes. These particles, made from a solid lipid core surrounded by a surfactant layer, are a more recent nanoDDS that have gained considerable interest in the last two decades in several research areas.[26,27] The application of LNP on parasitic diseases is still not widely used as liposomes but its value in improving the therapeutic efficiency of known antileishmanial and antimalaria agents has been already reported in previous studies using amphotericin B,[28] artemether[29] and curcuminoids.[30] Although Lopes et al.[25] reported the production of ORZ-containing LNP (LNP-ORZ) there are no studies concerning the *in vitro* and *in vivo* therapeutic evaluation of these particles.

The present study describes a systematic comparison of two lipid-based nanoformulations (liposomes and LNP) aiming at improving the *in vitro* and *in vivo* performance of ORZ as an antileishmanial agent. This includes, *in vitro* activity and toxicity and *in vivo* antileishmanial activity in a VL murine model.

MATERIAL AND METHODS

Materials

ORZ was purchased from Supelco (Bellefonte, USA), and pure phospholipids (dimyristoylphosphatidylcholine–DMPC and dimyristoylphosphoglycerol–DMPG) were supplied by Avanti Polar Lipids, Inc. (Alabaster, USA) and soya lecithin (Lipoid S100) from Lipoid (Ludwigshafen, Germany). Tripalmitin (glyceryl tripalmitate, purity $\geq 85\%$, melting point 66 °C), sodium deoxycholate and Tween® 20 were obtained from Sigma–Aldrich (Spain). Acetonitrile (HPLC grade) was from Merck. Culture media, foetal bovine serum (FBS) and antibiotics (penicillin–streptomycin) were purchased from Invitrogen Life Technologies (USA). Distilled water was of Milli-Q quality (Millipore, Bedford, MD, USA). All other reagents were of analytical grade and were used without further purification.

Preparation of Lip-ORZ

The incorporation of ORZ in liposomes was performed by the dehydration–rehydration method (DRV) with some modifications.[13,16,31] Briefly, the appropriate amounts of phospholipids (10 mM) and ORZ (0.5 or 1 mM) were dissolved in chloroform and dried on a Büchi rotary evaporator RE-111 (Büchi, Switzerland) until a homogeneous film was formed. The film was dispersed with water, and the resultant suspension was frozen and lyophilised overnight in a Modulyo freeze-dryer (Edwards, Germany). The lyophilised powder was rehydrated in two steps with citrate buffer (10 mM citrate, 145 mM NaCl, pH 5.5): first with 2/10 of the final volume followed by mild vortexing, at room temperature, and then, 30 min after, rehydration was completed with the addition of the remaining volume (8/10 of the final volume) of citrate buffer. The so formed liposomes were then down-sized using an extruder device (LipexBiomembranes, Canada) by sequential extrusion through polycarbonate filters ranging from 0.8 to

0.2 μm in pore size until the desired size was obtained. Non-incorporated ORZ was separated from the liposome dispersion by size exclusion chromatography in a PD-10 column (Bio-Rad Laboratories, California, USA) using citrate buffer as eluent. Incorporated ORZ was quantified by high-pressure liquid chromatography (HPLC), using the method described under 2.3.2. Unloaded liposomes were prepared as described above, with the exception that no ORZ was added. For *in vitro* and *in vivo* studies, as higher ORZ concentration are needed, the eluted liposomes were concentrated by ultra-centrifugation (250,000 g, 1 h 30 min) in a Beckman L8-60M ultracentrifuge (Beckman Instruments, Inc., USA) and suspended in citrate buffer.

Preparation of LNP-ORZ

The LNP were prepared by the emulsion–solvent evaporation method using tripalmitin as the lipid component and sodium deoxycholate, Tween® 20 and soya lecithin as co-surfactants.[25] Briefly, tripalmitin and lecithin were dissolved in dichloromethane (organic phase) and then added to the aqueous phase containing the Tween® 20 and sodium deoxycholate. The dispersion step was performed during a 1 min period of sonication (Branson Sonifier 250, Danbury, USA) followed by 3.5 min at 10,000 rpm using a Silverson High Speed Mixer L5M (Silverson Machines, Chesham, UK). The nanoparticle dispersion was then kept under stirring for approximately 4 h at room temperature until complete evaporation of the dichloromethane. When incorporating ORZ, the drug (2.2 or 4.4 mM) was added to the organic phase. Non-incorporated ORZ was separated from the LNP dispersion by size exclusion chromatography in a PD-10 column (Bio-Rad Laboratories, California, USA) using PBS pH 7.4 as eluent. Incorporated ORZ was quantified by HPLC, using the method described under 2.4.2.1. For *in vitro* and *in vivo* studies, as higher ORZ concentration are needed, the eluted LNP formulations were concentrated after centrifugation (4,000 g, 10 min, $\times$ 3) through a membrane concentrator (Amicon® Ultra-4 centrifugal filter units, 100 KDa MWCO, Millipore, Ireland) and suspended in PBS.

Characterisation of Nanoformulations

Size and Zeta Potential Measurements

Liposome and LNP mean diameter (Ø) and polydispersity index (Pdi) were determined by quasi-elastic laser light scattering in a Malvern Zetasizer Nano S (Malvern Instruments, UK). The zeta potential (surface charge, ξ) was determined using laser Doppler anemometry in a Nano Z (Malvern Instruments, UK). Samples were appropriately diluted for the measurements.

ORZ and Lipid Quantification

After disruption of nanoformulations with acetonitrile ORZ was quantified by a HPLC method. The HPLC system consisted of a System Gold (Beckman Instruments, Inc., USA), a Midas type 830 auto-sampler and a Diode-Array 168 detector (Beckman Instruments, Inc., USA). ORZ analysis was performed by UV detection at a fixed wavelength of 284 nm. The analytical column was a Nucleosil C18, 5 μm (150 $\times$ 4.6 mm) analytical column (Supelco, USA) eluted with a mobile phase consisting of acetonitrile with 0.1 M trifluoroacetic acid (TFA):water with 0.1 M TFA (60:40 (v/v)) at a flow rate of 1 mL/min. Samples and standards (0.5–30 μg/mL) were loaded in the column with a Midas type 830 auto-sampler with a 20 μL sample loop. Data were recorded using 32 Karat software, version 7.0.

Phospholipid determination of liposomes was performed as described by Rouser et al.[32] based on the quantification of inorganic phosphorous. Tripalmitin and soya lecithin concentrations on LNP were determined by the methods of GPO-POD (glycerol phosphate dehydrogenase-Peroxidase) and Trinder, respectively, using enzymatic colorimetric assay kits (Spinreact, Girona, Spain).

Incorporation Efficacy and Drug Loading

ORZ nanoformulations were evaluated in terms of incorporation efficacy (I.E.) and drug loading capacity (L.C.) determined as described by the equations below.

$$\text{IE.} = ([\text{ORZ}]f/[\text{Lip}]f)/[\text{ORZ}]i/[\text{Lip}]i) \times 100$$

$$\text{L}\ \text{mol}) = [\text{ORZ/Lip}]f$$

where $[\text{ORZ}]f$ and $[\text{Lip}]f$ represent ORZ and lipid concentration in the final nanoformulations and $[\text{ORZ}]i$ and $[\text{Lip}]i$ represent ORZ and lipid concentration in the initial suspensions.

Cells and Culture Conditions

L. infantum promastigotes (MHOM/MA/67/ITMAP-263) kindly provided by Professor Ana Tomás (IBMC, Universidade do Porto) were grown in RPMI-1640 supplemented with 10% FBS, antibiotics and 25 mM HEPES, pH 7.4 at 26 °C. The virulence of *Leishmania* parasites was maintained by passage in BALB/c mice. For *in vitro* studies, *L. infantum* promastigotes were transfected by electroporation as previously described[33] to obtain promastigotes expressing green fluorescent protein (GFP). The human monocytic leukaemia THP-1 cell line was used as a host for leishmania parasites. The THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS, 1 mM HEPES, 2 mM glutamine, antibiotics and 1 mM sodium pyruvate, pH 7.2 at 37 °C with 5% CO_2.

Animals

Balb/c male mice (22–24 g) were obtained from Charles River, Barcelona, Spain. Animals were fed with standard laboratory food and water *ad libitum*. All experimental animal procedures were carried out with the permission of the local animal ethical committee and licensed by Direção

Geral de Alimentação e Veterinária (DGAV). All animals were handled in strict accordance with good animal practice under the Declaration of Helsinki, the EEC Directive (86/609/EEC) and the Portuguese laws D.R. no. 31/92, D.R. 153 I-A 67/92, and all following legislations.

Biological Evaluation of ORZ Nanoformulations

Haemolytic Potential

The haemolytic activity was determined using EDTA-preserved peripheral human blood, according to Esteves et al.[34] Briefly, blood was centrifuged to remove the plasma, and the red blood cells (RBCs) were washed three times in PBS. After the final wash, RBCs were distributed in 96-well microplates (100 μL/well) and equal volumes of Lip-ORZ, LNP-ORZ and Free-ORZ (both diluted in PBS in concentrations between 4 and 500 μM) were added. After incubation at 37 °C for 1 h, the plates were centrifuged (800 g, 10 min) and the supernatants recovered. The absorbance of the supernatant was measured at 540 nm with the reference filter at 620 nm in a microplate reader (ELx800, Biotek, USA). In each plate negative and positive controls were prepared by incubating RBCs with PBS and water, respectively. The absorbance of Lip-ORZ, LNP-ORZ and Free-ORZ solutions was also determined and used as control. The percentage of haemolytic activity of each formulation at different concentrations was estimated using $[(A - A_0)/(A_{max} - A_0)] \times 100$, where A_0 is the negative control haemolysis and A_{max} corresponds to 100% haemolysis (positive control). The haemolytic activity was also evaluated by the determination of HC_{50} value (drug concentration that lyses 50% of RBCs) calculated by linear regression analysis.

Cytotoxicity

Studies were carried out using the THP-1 human monocytic cell line as described by Plano et al.[35] A quantitative measurement of the cell damage after incubation with different concentrations of ORZ (Lip-ORZ, LNP-ORZ and Free-ORZ) was evaluated by flow cytometry using the propidium iodide (PI) exclusion method. The THP-1 cells during logarithmic growth phase (4×10^5 cells/mL) were incubated with concentrations of Lip-ORZ, LNP-ORZ and Free-ORZ ranging from 0.04 to 25 μM. After an incubation period of 24 h (37 °C, 5% CO_2), cells were stained with PI (5 μg/mL) and then analysed for PI fluorescence by flow cytometry. The cytotoxicity was evaluated by the determination of CC_{50} value (drug concentration that reduced the percentage of viable cells in 50%) calculated by linear regression analysis.

In Vitro Antileishmanial Activity in Promastigote Cultures

In vitro activity in promastigotes cultures was determined using *L. infantum* promastigotes expressing GFP, according to Plano et al.[35] *L. infantum* promastigotes expressing GFP during the logarithmic growth phase (2×10^5 cells/mL) were incubated with different concentrations of Lip-ORZ, LNP-ORZ and Free-ORZ (0.04–25 μM) in 96-well tissue culture plates (Cellstar, Greiner) at 26 °C for 24 h. The percentage of viable promastigotes was evaluated by flow cytometry using the PI exclusion method. The activity was evaluated by the determination of IC_{50} value (drug concentration that reduced the percentage of viable promastigotes in 50%) calculated by linear regression analysis.

In Vitro Antileishmanial Activity Against the Intracellular L. infantum

Intracellular activity was determined according to Plano et al.[35] Briefly, THP-1 cells were seeded in 24 well plates (Nunc, Roskilde, Denmark) and differentiated to macrophages for 24 h in 1 mL of RPMI-1640 medium containing 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA). Culture medium was removed and the macrophages were infected with promastigotes expressing GFP in 1 mL of THP-1 medium (promastigote:macrophage ratio, 5:1) for 5 h at 37 °C, 5% CO_2. After incubation, non-infecting promastigotes were removed; plates were washed two times with 1× PBS and replaced with new RPMI-1640 medium containing different concentrations Lip-ORZ, LNP-ORZ and Free-ORZ (10–50 μM). After 72 h treatment, medium was removed; THP-1 cells were washed one time with 1× PBS and detached with TrypLE™ Express (Invitrogen, Leiden, the Netherlands) according to the manufacturer's indications. Cells were stained with PI and the infection quantification was measured by flow cytometry. The antileishmanial effect was evaluated by the determination of IC_{50} value (drug concentration that reduced infection in 50%) calculated by linear regression analysis.

Flow Cytometry Analysis

Cell samples were analysed on a Guava® easyCyte™ 5HT flow cytometer and InCyteTM software (Millipore, Bedford, MD, USA), equipped with a 488 nm argon laser. At least 5,000 cells were analysed per sample, and data analysis were performed on fluorescence intensities (GFP: 545 nm and PI: 645 nm) that excluded cell autofluorescence and cell debris.

In Vivo Antileishmanial Activity

The animals were infected by intravenous (i.v.) injection in a lateral tail vein with 200 μL of an *L. infantum* inoculum with 10^6 promastigotes (MHOM/MA/67/ITMAP-263).[36] The treatment started one week post-infection (day 7) when the animals were randomly sorted into groups of five mice. Free-ORZ was prepared in a mixture of citrate buffer and Tween® 80 (95/5% w/w).

Study of the effect of administration route: mice were treated with either Lip-ORZ or Free-ORZ (25 mg

ORZ/kg/day) by i.v. route of administration for 5 consecutive days (days 7–11).

Study of the effect of number of administrations: mice were treated with Lip-ORZ, LNP-ORZ and Free-ORZ (25 mg ORZ/kg/day) by i.v. route of administration for 5 (days 7–11) or 10 (day 7–11 and 14–18) consecutive days with two days interval in-between.

Dose-response relationship: mice were treated with 6.25, 12.5 and 25 mg ORZ/kg/day by i.v. route of administration for 5 consecutive days (days 7–11). ORZ was administered as Lip-ORZ, LNP-ORZ or Free-ORZ.

Study of the effect of lipid excipient: mice were treated with 6.25 mg ORZ/kg/day with different lipid concentrations by i.v. route of administration for 5 consecutive days (days 7–11). ORZ was administered as Lip-ORZ or LNP-ORZ. As a control, unloaded liposomes and LNP (equivalent dose of lipid) were also administered i.v. following the same treatment regimen as the ORZ nanoformulations.

Regardless of the administration route, all nanoformulations were administered in 0.2 mL final volume. In i.v. treatment, ORZ nanoformulations and Free-ORZ were administered in the lateral tail vein.

Evaluation of Parasitic Burden (PB) in Mice

In all treatment regimens above described animals were sacrificed by cervical dislocation 3 days after the last treatment administration, and spleens and livers were aseptically collected and weighted. Viable PB was determined by the limiting dilution assay (LDA). Briefly, both organs were homogenized in Schneider's Drosophila medium supplemented with 10% heat-inactivated FBS, and then diluted in the same medium to a final concentration of 10 mg/mL. These cell suspensions were then titrated in quadruplicate across a 96-well plate in serial four-fold dilutions (four titrations per organ). After 10 days of growth at 26 ± 1 °C, the last dilution containing promastigotes was recorded and the number of parasites per gram of organ (parasite burden) calculated as described by Buffet et al.[37]

The suppression of parasite growth was calculated as the percentage inhibition relative to parasitic burden of negative control animals:

$$PB_{reduction}(\%) = [1 - (PB_{Treated}/PB_{Control})] \times 100$$

where $PB_{Treated}$ represented the PB of the treated animal and $PB_{Control}$ the average PB of the negative control group.

The PB growth index (GI) was calculated using the following formula:

$$\text{PB Growth index (GI)} = (\log_{10} PB_{\text{end of treatment}} - \log_{10} PB_{\text{beginning of treatement}})$$

Statistical Analysis

Data presented are expressed as mean ± standard deviation (SD), as mentioned in legends of figures and tables. Statistical analysis was performed using one-way ANOVA, with the exception of the data obtained in the *in vivo* studies which were analysed with a Kruskal-Wallis test with Dunn's post-test. Differences were considered significant with a 5% significance level ($p < 0.05$). Curve fitting for dose-response curves was carried out using GraphPad 5 (GraphPad Software Inc., San Diego, California).

RESULTS

Characterization of ORZ Nanoformulations

In previous published work the optimization of the best experimental conditions for the incorporation of ORZ in liposomes and LNP was established and pharmaceutically acceptable formulations were achieved.[13,25] The incorporation parameters and physicochemical properties of the ORZ nanoformulations (liposomes and LNP) used in all *in vitro* and *in vivo* studies in this report are displayed in Table I. Regardless of differences in their structure, chemical composition and preparation methods, both nanoformulations present high ORZ loadings and high I.E. without significant statistical differences ($p > 0.05$). In addition both nanoformulations present a negative zeta potential and a similar granulometric distribution ($p > 0.05$) suitable for parenteral administration. The incorporation of ORZ did not affect the properties of the nanoformulations as unloaded ones exhibit similar results for particle size or zeta potential ($p > 0.05$).

In Vitro Biological Evaluation

ORZ nanoformulations and Free-ORZ were evaluated *in vitro* for their toxicity to THP-1 human monocytic leukaemia cell line (macrophage like cell line), haemolytic activity to RBCs and antileishmanial activity. The antileishmanial activity was evaluated against *L. infantum* promastigote cultures as well as against the intracellular amastigote form using infected THP-1 cells.

Haemolytic Activity and Cytotoxicity

The evaluation of the *in vitro* potential adverse effects of ORZ formulations revealed that Free-ORZ exhibits haemolytic activity against RBCs (HC_{50} of 425 μM) and also cytotoxicity for THP-1 cells (CC_{50} of 20 μM) (Table II). In contrast none of the ORZ nanoformulations evidenced either haemolytic activity or cytotoxic effects up to the highest concentrations tested (500 μM and 25 μM, respectively).

Activity Against Promastigotes and Intracellular Form of L. infantum

The *in vitro* antileishmanial activity assays showed that Free-ORZ was active against both the promastigote ($IC_{50} = 17$ μM) and the intracellular amastigote stage ($IC_{50} = 19$ μM) of the parasite (Table II). As for the nanoformulations it was found that ORZ antileishmanial activity decreased against both parasite forms. This might be due to the less availability of ORZ to the culture

Table I. Incorporation parameters and physicochemical properties of different ORZ nanoformulations.

Formulation	Composition[a] (molar ratio)	L.C. g/mol (% w/w)	I.E. (%)	Ø (μm)	Pdi	Zeta potential (mV)
Liposomes	DMPC:DMPG (7:3)	–	–	0.22 ± 0.02	0.20 ± 0.01	− 35 ± 3
	DMPC:DMPG:ORZ (7:3:0.5)	16 ± 2 (2.3 ± 0.3)	94 ± 3	0.21 ± 0.03	0.19 ± 0.02	− 32 ± 2
	DMPC:DMPG:ORZ (7:3:1)	32 ± 1 (4.7 ± 0.1)	91 ± 2	0.22 ± 0.02	0.20 ± 0.01	− 30 ± 1
LNP	Lecit:Tripal (7.3:2.4)	–	–	0.16 ± 0.02	0.25 ± 0.01	− 23 ± 4
	Lecit:Tripal:ORZ (7.3:2.4:0.5)	17 ± 1 (2.4 ± 0.1)	97 ± 2	0.17 ± 0.03	0.26 ± 0.01	− 24 ± 1
	Lecit:Tripal:ORZ (7.3:2.4:1)	34 ± 2 (4.8 ± 0.3)	95 ± 2	0.17 ± 0.02	0.25 ± 0.02	− 22 ± 3

Notes: Data are expressed as mean ± SD ($n = 3$); Lecit: Lecithin; Tripal: Tripalmitin; [a]ratio presented as μmol:μmol.

medium as it is incorporated in nanoDDS. This behaviour was observed for both nanoformulations.

In Vivo Evaluation

The therapeutic activity of ORZ nanoformulations was evaluated in a murine model of VL to establish the best treatment regimens, including: number of administrations, ORZ dose-response relationship and influence of the lipid excipient. For these purposes Lip-ORZ, LNP-ORZ and Free-ORZ were administered under different conditions to Balb/c mice infected with *L. infantum*. The PB was determined and compared to infected and untreated animals.

Comparison with Glucantime®

Figure 1 shows the PB in the liver and spleen of infected mice after 5 administrations of Lip-ORZ, Free-ORZ and a commercially available antileishmanial drug (Glucantime®) administrated under standard conditions described in the literature.[38] Glucantime® and Lip-ORZ presented similar results in spleen, whereas in the liver Lip-ORZ induced a 97% PB reduction as compared to 81% for Glucantime® even using a 2 fold higher administered dose (Fig. 1).

Comparative Study of Lip and LNP-ORZ Nanoformulations

The therapeutic activities of both nanoformulations with Free-ORZ in liver and spleen were compared. The results displayed in Figure 2(A) show significant PB reductions ($p < 0.05$) in the liver (89 and 84%) and spleen (84 and 91%) for Lip-ORZ and LNP-ORZ, respectively as compared to control group proving that both ORZ nanoformulations are highly efficient in the treatment of infected mice. For Free-ORZ we observed a significant PB reduction ($p < 0.05$) only in the spleen while no significant reduction was found in the liver. The therapeutic activity was also evaluated by calculating the growth index (GI), which represents the difference between the PB at the end and at the beginning of treatment (Fig. 2(B)). The negative GI values obtained in the liver (− 0.49 for Lip-ORZ and − 0.33 for LNP-ORZ) and in the spleen (− 0.47 for Lip-ORZ and − 0.69 for LNP-ORZ) indicate that ORZ nanoformulations were able to reduce the parasitic infection to values lower than those observed before treatment. Although to a lesser extent, Free-ORZ also induced a reduction on the parasitic infection as shown by the respective negative GI values (liver: − 0.05; spleen: − 0.28).

Table II. *In vitro* biological evaluation of ORZ nanoformulations and Free-ORZ.

Formulation	Haemolysis HC_{50} (μM)	Cytotoxicity CC_{50} (μM)	Promastigotes *L. infantum* IC_{50} (μM)	Intracellular *L. infantum* IC_{50} (μM)
Free-ORZ	425 ± 10	20 ± 5	17 ± 1	19 ± 2
Lip-ORZ	> 500	> 25	> 25	48 ± 3
LNP-ORZ	> 500	> 25	> 25	44 ± 2

Notes: Data are expressed as mean ± SD ($n = 3$); Lip-ORZ: ORZ liposomes; LNP-ORZ: ORZ solid lipid nanoparticles.

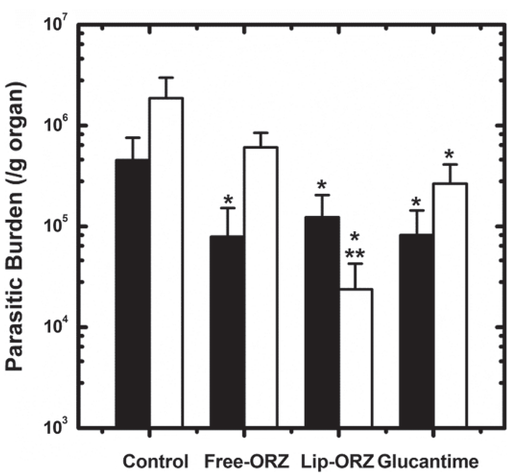

Figure 1. Effect of treatment of Balb/c mice infected with *L. infantum*. Mice were treated with 5 consecutive i.v. administrations of Lip-ORZ and Free-ORZ (25 mg/kg/day) or 5 consecutive subcutaneous (s.c.) injections of Glucantime® (45 mg/kg/day). Data are expressed as PB per gram of spleen (■) and liver (□); mean ± SD ($n = 5$). *$p < 0.05$ as compared to control; **$p < 0.05$ as compared to Free-ORZ.

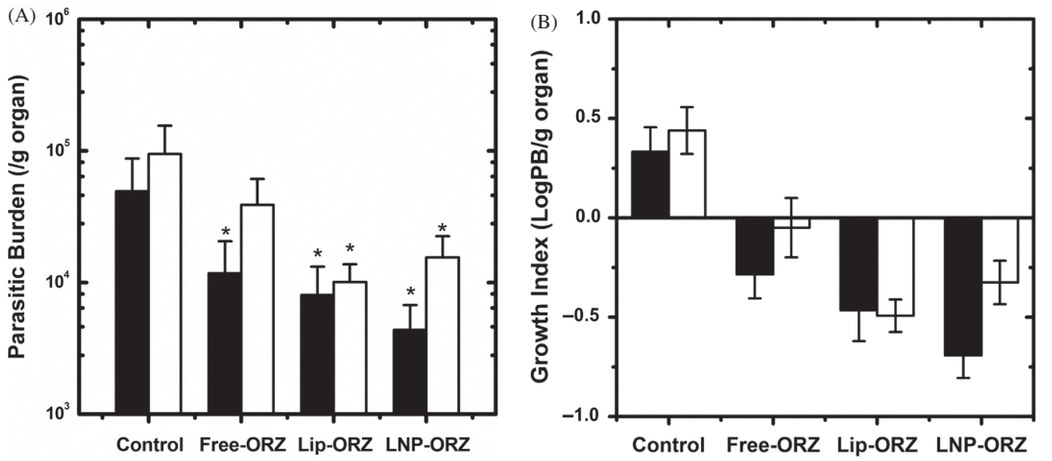

Figure 2. **Effect of 5 administrations on the PB (A) and GI (B) in the spleen (■) and in the liver (□) of Balb/c mice infected with *L. infantum*. Infected mice were treated with 5 consecutive i.v administrations of Lip-ORZ, LNP-ORZ or Free-ORZ (25 mg/kg/day); mean ± SD ($n = 5$). *$p < 0.05$ as compared to control.**

Effect of Number of Administrations

In order to determine if the therapeutic activity of ORZ formulations could be further improved we increased the number of administrations from 5 to 10. For this purpose we evaluated the PB in the liver and spleen after administration of Lip-ORZ and LNP-ORZ and Free-ORZ (Figs. 3(A) and (B)).

Results show a significant and continuous increase in PB for the control group during the treatment period. The same PB evolution was observed for Free-ORZ in the liver, indicating that this formulation is not effective in fighting the infection for the same period. With respect to the nanoformulations, a significant reduction in PB was observed as compared to control in both organs and also for Free-ORZ in the spleen, regardless the number of administrations. Both nanoformulations and treatment schedules not only repressed PB growth but also reduced it to levels below those observed in the beginning of treatment. However, we expected that the 10 administrations would further decrease the PB of treated groups. As this was not observed the shorter treatment regimen was chosen for further studies as this schedule was less distressful for animals and less cost-effective.

Dose-Response Study

The ORZ dose-response curves for PB reduction in liver and spleen after 5 days treatment with ORZ nanoformulations and Free-ORZ are shown in Figures 4(A) and (B). The dose response curves for both ORZ nanoformulations, reached a plateau between 6.25 mg/kg and 12.5 mg/kg, either in liver or in spleen. However, different behaviours for each formulation in each organ were

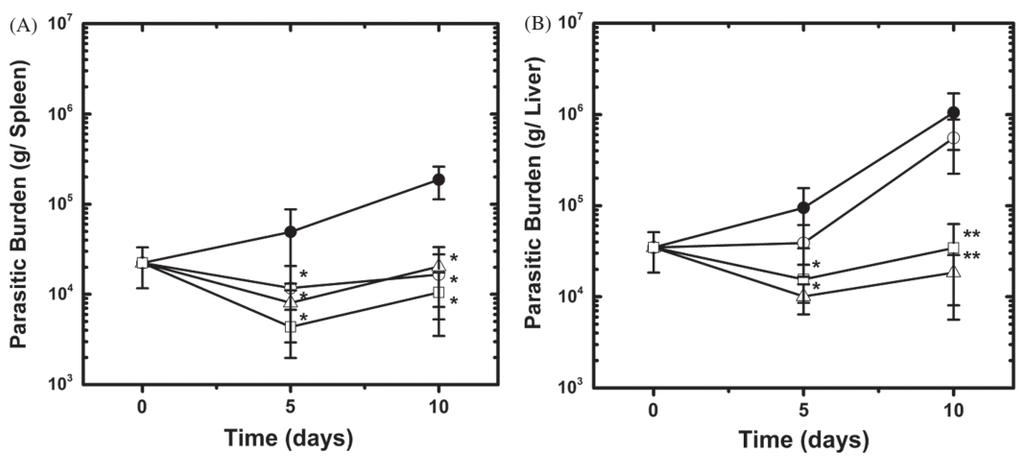

Figure 3. **Effect of number of administrations on the PB in the spleen (A) and liver (B) of Balb/c mice infected with *L. infantum*. Infected mice were treated with 5 or 10 consecutive i.v administrations of Lip-ORZ (Δ), LNP-ORZ (□) or Free-ORZ (○) (25 mg/kg/day); control mice (•); mean ± SD ($n = 5$). *$p < 0.05$ as compared to control and **$p < 0.05$ as compared to control and Free-ORZ.**

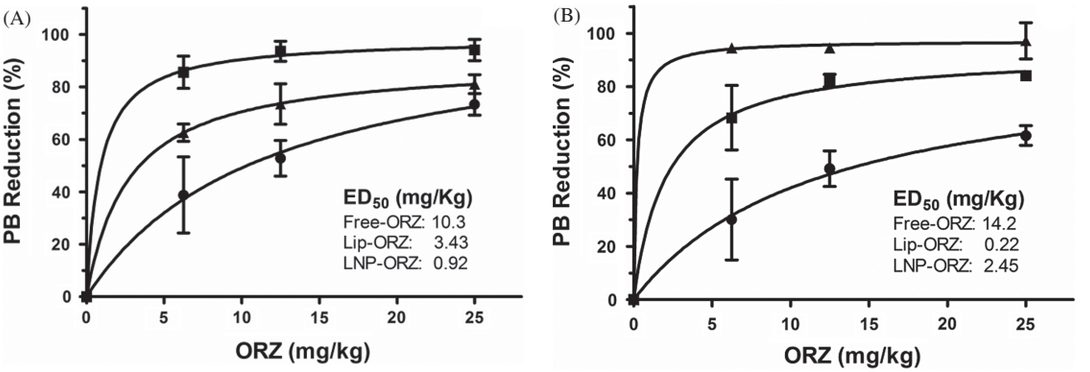

Figure 4. **ORZ dose-response curves in terms of PB in spleen (A) and in liver (B) of Balb/c mice infected with *L. infantum*. Mice were treated with 5 consecutive i.v. administrations of Lip-ORZ (▲), LNP-ORZ (■) or Free-ORZ (•) (6.25, 12.5 and 25 mg/kg/day) on consecutive days. Data are expressed as the PB reduction per gram of spleen and liver as compared to control; mean ± SD of 2 combined experiments ($n = 10$).**

observed. In fact, LNP-ORZ resulted in a higher PB reduction than Lip-ORZ, in the spleen with a plateau at 6.25 mg/kg (86%), while for Lip-ORZ a plateau was only reached at 12.5 mg/kg (74%) (Fig. 4(A)). In the liver a reverse situation was observed with a plateau at 6.25 mg/kg (95%) for Lip-ORZ and only at 12.5 mg/kg (82%) for LNP-ORZ (Fig. 4(B)). Compared to the results obtained with ORZ nanoformulations, Free-ORZ elicited the lowest PB reduction for all ORZ doses tested. The increase of the administered dose from 6.25 mg/kg to 25 mg/kg gave rise to a concomitant reduction on the PB, reaching a maximum of 73% and 62% in spleen and in liver, respectively. Even at 25 mg/kg, Free-ORZ PB reduction was lower than the obtained with both nanoformulations at 6.25 mg/kg or 12.5 mg/kg. This means that Lip-ORZ and LNP-ORZ are more active than Free-ORZ as observed by comparing the ED_{50} values of all formulations (Figs. 4(A) and (B)).

Effect of Lipid Excipient

To clarify the possible effect of the lipid excipient on the ORZ therapeutic activity, nanoformulations with different lipid contents, for the same ORZ dose (6.25 mg/kg) were tested (Table III). No significant differences ($p > 0.05$) on the PB were found between control and groups treated with unloaded liposomes and LNP. Moreover, using different lipid doses and the same ORZ dose no significant differences ($p > 0.05$) were observed on the PB in liver and in spleen. These results indicate that the antileishmanial effect arises from ORZ and not from the lipid carriers and also that this excipient does not interfere with the drug activity.

Table III. **Effect of the lipid excipient on the Log_{10} PB of Balb/c mice infected with *L. infantum*.**

		Log_{10} (PB)	
	Lipid (mg/kg)	Spleen	Liver
Control	–	5.4 ± 0.2	5.7 ± 0.3
Unloaded liposomes	142	5.3 ± 0.2	5.7 ± 0.2
Lip-ORZ	142	4.7 ± 0.2*	4.7 ± 0.2*
	262	4.9 ± 0.1*	4.8 ± 0.2*
Control		5.2 ± 0.2	6.4 ± 0.3
Unloaded LNP	132	5.2 ± 0.2	6.4 ± 0.3
LNP-ORZ	132	4.2 ± 0.2*	5.6 ± 0.2*
	259	4.5 ± 0.2*	5.7 ± 0.2*

Notes: Data are expressed as Log_{10} (PB) per gram of organ; mean ± SD ($n = 5$). *$p < 0.05$ to control and to unloaded liposomes or LNP.

DISCUSSION

The increasing interest in dinitroanilines as chemotherapeutic agents is documented by the number of reports describing their *in vitro* antileishmanial activity. However the *in vivo* evaluation of these agents is rather scarce either for dinitroanilines in free form or incorporated in nanoDDS. We were the first to report the *in vivo* antileishmanial activity of liposomal TFL, a dinitroaniline incorporated in liposomes, in a *L. donovani* murine model[23] and in an experimental canine leishmaniasis.[24] More recently, ORZ, another dinitroaniline was incorporated in two lipid-based nanoDDS (Lip-ORZ and LNP-ORZ). These nanoformulations were characterized in terms of their physicochemical properties and biologically evaluated (haemolytic activity, toxicity towards macrophages).[13,25] The biodistribution of Lip-ORZ was also performed in healthy animals, proving that the incorporation in liposomes is essential for targeting liver and spleen.[13] At this stage it is crucial to evaluate the *in vitro* and more important the *in vivo* therapeutic efficacy of Lip-ORZ and LNP-ORZ as possible nanopharmaceuticals against leishmaniasis, while comparing systematically the two nanoDDS as ORZ carriers.

As both nanoformulations were intended for parenteral administration all materials used in the preparation of Lip-ORZ and LNP-ORZ are pharmaceutically acceptable for this purpose.[39] In fact most of them are currently used in several commercialized parenteral formulations,

including those for the treatment of leishmaniasis such as Fungizone® (sodium deoxycholate) or Abelcet® (DMPC and DMPG).

Another important aspect kept in mind was the control of particle size distribution and zeta potential, not only to allow their correct comparison, but also to meet the purpose of the study, as these characteristics will define *in vitro* and *in vivo* behaviour of ORZ nanoformulations. The size range chosen (160–220 nm) not only is suitable for parenteral administration but also for the uptake by the liver and spleen macrophages.[40] The highly negative zeta potential of both nanoformulations, obtained by a suitable lipid composition (high contents of negative phospholipids and surfactants), will also favour macrophage uptake, as intended.[40] Moreover the negative surface charge is expected to play an important role on nanoformulations stability by preventing particle aggregation due to electrostatic repulsion.[41]

In spite of differences in raw materials and preparation methods between Lip-ORZ and LNP-ORZ they were very similar in the ability to incorporate ORZ with high I.E. ($>90\%$) and L.C. (≈ 30 g ORZ/mol of lipid or $\approx 5\%$ w/w). As demonstrated in our previous studies, ORZ is incorporated within the lipid bilayer of liposomes,[13] whereas in LNP is incorporated both in the lipid matrix and in the surfactant layer surrounding it.[25] In both cases ORZ incorporation had no significant effect on size and zeta potential of the nanoformulations (Table I).

After optimization of ORZ nanoformulations we proceeded to verify whether the incorporation was translated into an improvement of drug performance. This was confirmed by the reduction on the cytotoxicity and the absence of haemolytic activity after ORZ incorporation, demonstrating a rather protective role of nanoformulations to mammalian cells. Similar results were obtained with other antileishmanial drugs incorporated either in liposomes[42, 43] or LNP.[28] These protective features may also explain the lower activity of Lip-ORZ and LNP-ORZ, observed against *L. infantum* promastigotes, since after incorporation ORZ is less available to interact with the parasite in culture. Nevertheless, incorporated ORZ retained its activity against intracellular amastigotes, the clinically relevant form of the parasite.

The comparative studies of ORZ nanoformulations was focused on the *in vivo* evaluation of therapeutic activity as *in vitro* studies are not always predictive of biological performance. Relevant aspects, such as number of administrations, ORZ dose-response relationship and effect of lipid excipient were explored in a murine model of VL. Among the parenteral routes of administration, i.v. was selected as it is one of the most widely used, in murine models, for the administration of antileishmanial drugs and nanoDDS.[44, 45] Previous works have shown that the distribution of liposomes and LNP to liver and spleen was higher after i.v. as compared to i.p. and even s.c. administration.[46, 47] Using this route of administration, the efficacy of Lip-ORZ was demonstrated as compared to Glucantime® even at lower doses (Fig. 1). Using the same treatment conditions, liposomes and LNP proved to enhance ORZ activity in the liver and spleen as indicated by an up to 3 fold PB reduction. This enhancement correlates well with the higher accumulation of ORZ nanoformulations observed in these organs as we demonstrated before.[13] While this was observed for nanoformulations with a particle size around 140 nm, in the present study ORZ nanoformulations accumulation is expected to be higher as it concomitantly increases with the particle size (160–220 nm), as described before.[48–50]

Our strategy to further reduce PB by increasing the number of administrations with ORZ nanoformulations from 5 to 10 was not translated into an improved therapeutic effect (Fig. 3). In fact 10 administrations did not lead to a further reduction in the PB as expected. Results show that ORZ nanoformulations were only able to arrest the PB increase observed in the control group, maintaining the values obtained after 5 administrations. This suggests that the concentration of ORZ present in the liver and spleen did not concomitantly increase with the number of administrations, probably due to hepatic and splenic saturation as described before.[51, 52]

The dose-response curves clearly demonstrate the improvement obtained by incorporation of ORZ in nanoformulations by a strong reduction of ED_{50} values in both organs as compared to Free-ORZ. Reductions of 65 (Lip-ORZ) and 6 (LNP-ORZ) fold for liver and 3 (Lip-ORZ) and 11 (LNP-ORZ) fold for spleen were observed. This experiment also allowed concluding that lower doses of incorporated ORZ are enough to attain the maximal activity, as a plateau was reached between 6.25 and 12.5 mg/kg. The lowest dose of incorporated ORZ presented an activity similar to that of a 4 fold higher dose of Free-ORZ, confirming the superiority of the nanoformulated drug.

It is interesting to observe that each nanoformulation presents a different activity profile in each target organ. While higher activity was observed for LNP-ORZ in the spleen (4 fold lower ED_{50}), Lip-ORZ was more active in the liver (11 fold lower ED_{50}). As size and surface charge characteristics of both nanoformulations were similar, as well as the protocol for therapeutic activity evaluation, the results suggest differences in the biodistribution of the nanoformulations in these organs due to some other factors. One hypothesis could be the different rigidity of both nanoformulations, as Lip-ORZ presents a phase transition temperature of $+23$ °C, whereas LNP presents a lipid core of tripalmitin with a melting point of $+62$ °C.[13, 25] In physiological conditions the liposomal bilayer will be in a fluid state while LNP remain in a solid state and thus less flexible. This rigidity/inflexibility of LNP will probably lead to a high retention in spleen, as the width of interendothelial cell slits in the spleen venous is approximately 200–250 nm.[53, 54] The flexibility of Lip-ORZ makes them

deformable enough to overcome splenic filtration. This hypothesis will be elucidated in near future after appropriate biodistribution profile evaluation.

It was clarified that the antileishmanial activity is due to ORZ and not to the lipid excipients, as demonstrated by absence of any activity for the unloaded nanoformulations. Similar observations for lipid-based systems have been already reported in the literature.[55, 56]

CONCLUSIONS

The incorporation of ORZ either in liposomes or LNP with suitable physicochemical properties for *in vivo* administration allowed reducing the cytotoxicity and abolished the haemolytic activity evidenced by Free-ORZ. The ORZ nanoformulations also kept the antileishmanial intracellular activity. *In vivo* studies demonstrated that incorporation of ORZ in liposomes and in LNP clearly improved its pharmacological performance. There were no significant differences between both nanoformulations, except for the preferential activity found in each target organs. In future work, biodistribution profiles should be performed to elucidate this behaviour.

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REFERENCES

1. World Health Organization, (ed.), Expert Committe on the Control of Leishmaniases, Control of the leishmaniases: WHO Techinical Report Series 949, WHO Press, Geneve **(2010)**.
2. E. Handman, Leishmaniasis: Current status of vaccine development. *Clin. Microbiol. Rev.* 14, 229 **(2001)**.
3. L. Kedzierski, A. Sakthianandeswaren, J. M. Curtis, P. C. Andrews, and P. C. Junk, Leishmaniasis: Current treatment and prospects for new drugs and vaccines. *Curr. Med. Chem.* 16, 599 **(2009)**.
4. P. Le Pape, Development of new antileishmanial drugs-current knowledge and future prospects. *J. Enzym. Inhib. Med. Chem.* 23, 708 **(2008)**.
5. B. J. Fennell, J. A. Naughton, J. Barlow, G. Brennan, I. Fairweather, E. Hoey, N. McFerran, A. Trudgett, and A. Bell, Microtubules as antiparasitic drug targets. *Expert. Opin. Drug Discov.* 3, 501 **(2008)**.
6. Y. M. Traub-Cseko, J. M. Ramalho-Ortigão, A. P. Dantas, S. L. de Castro, H. S. Barbosa, and K. H. Downing, Dinitroaniline herbicides against protozoan parasites: The case of *Trypanosoma cruzi*. *Trends parasitol.* 17, 136 **(2001)**.
7. A. Armson, S. W. Kamau, F. Grimm, J. A. Reynoldson, W. M. Best, L. M. MacDonald, and R. C. Thompson, A comparison of the effects of a benzimidazole and the dinitroanilines against *Leishmania infantum*. *Acta Trop.* 73, 303 **(1999)**.
8. M. M. Chan, J. Tzeng, T. J. Emge, C. T. Ho, and D. Fong, Structure-function analysis of antimicrotubule dinitroanilines against promastigotes of the parasitic protozoan *Leishmania mexicana*. *Antimicrob. Agents Chemother.* 37, 1909 **(1993)**.
9. G. Bhattacharya, M. M. Salem, and K. A. Werbovetz, Antileishmanial dinitroaniline sulfonamides with activity against parasite tubulin. *Bioorg. Med. Chem. Lett.* 12, 2395 **(2002)**.
10. B. J. Fennell, J. A. Naughton, E. Dempsey, and A. Bell, Cellular and molecular actions of dinitroaniline and phosphorothioamidate herbicides on *Plasmodium falciparum*: Tubulin as a specific antimalarial target. *Mol. Biochem. Parasitol.* 145, 226 **(2006)**.
11. M. M. Chan and D. Fong, Inhibition of leishmanias but not host macrophages by the antitubulin herbicide trifluralin. *Science* 249, 924 **(1990)**.
12. European Food Safety, and Authority, Conclusion on the peer review of the pesticide risk assessment of the active substance oryzalin. *EFSA Scientific Report* 8, 1 **(2010)**.
13. R. M. Lopes, M. L. Corvo, C. V. Eleutério, M. C. Carvalheiro, E. Scoulica, and M. E. M. Cruz, Formulation of oryzalin (ORZ) liposomes: *In vitro* studies and *in vivo* fate. *Eur. J. Pharm. Biopharm.* 82, 281 **(2012)**.
14. S. Stegemann, F. Leveiller, D. Franchi, H. de Jong, and H. Lindén, When poor solubility becomes an issue: From early stage to proof of concept. *Eur. J. Pharm. Sci.* 31, 249 **(2007)**.
15. M. E. M. Cruz, S. I. Simões, M. L. Corvo, M. B. F. Martins, and M. M. Gaspar, In Drug Delivery Nanoparticles Formulation and Characterization, edited by Y. Pathak and D. Thassu, Informa Healthcare USA, Inc., New York **(2009)**, Vol. 191, pp. 35–50.
16. M. M. Gaspar, A. Cruz, A. F. Penha, J. Reymão, A. C. Sousa, C. V. Eleutério, S. A. Domingues, A. G. Fraga, A. L. Filho, and M. E. M. Cruz, Rifabutin encapsulated in liposomes exhibits increased therapeutic activity in a model of disseminated tuberculosis. *Int. J. Antimicrob. Agents* 31, 37 **(2008)**.
17. C. R. Alving, E. A. Steck, W. L. Chapman, V. B. Waits, L. D. Hendricks, G. M. Swartz, and W. L. Hanson, Therapy of leishmaniasis: Superior efficacies of liposome-encapsulated drugs. *Proc. Natl. Acad. Sci. USA* 75, 2959 **(1978)**.
18. A. Papagiannaros, C. Bories, C. Demetzos, and P. M. Loiseau, Antileishmanial and trypanocidal activities of new miltefosine liposomal formulations. *Biomed. Pharmacother.* 59, 545 **(2005)**.
19. J. P. Gangneux, A. Sulahian, Y. J. Garin, R. Farinotti, and F. Derouin, Therapy of visceral leishmaniasis due to *Leishmania infantum*: Experimental assessment of efficacy of AmBisome. *Antimicrob. Agents Chemother.* 40, 1214 **(1996)**.
20. A. G. Tempone, R. A. Mortara, H. F. de Andrade, and J. Q. Reimão, Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis. *Int. J. Antimicrob. Agents* 36, 159 **(2010)**.
21. V. Vanlerberghe, G. Diap, P. J. Guerin, F. Meheus, S. Gerstl, P. Van der Stuyft, and M. Boelaert, Drug policy for visceral leishmaniasis: A cost-effectiveness analysis. *Trop. Med. Int. Health* 12, 274 **(2007)**.
22. S. L. Croft, S. Sundar, and A. H. Fairlamb, Drug resistance in leishmaniasis. *Clin. Microbiol. Rev.* 19, 111 **(2006)**.
23. M. Carvalheiro, J. Jorge, C. Eleutério, A. F. Pinhal, A. C. Sousa, J. G. Morais, and M. E. M. Cruz, Trifluralin liposomal formulations active against *Leishmania donovani* infections. *Eur. J. Pharm. Biopharm.* 71, 292 **(2009)**.
24. C. Marques, M. Carvalheiro, M. A. Pereira, J. Jorge, M. E. M. Cruz, and G. M. Santos-Gomes, Efficacy of the liposome trifluralin in the treatment of experimental canine leishmaniosis. *Vet. J.* 178, 133 **(2008)**.
25. R. Lopes, C. V. Eleutério, L. M. D. Gonçalves, M. E. M. Cruz, and A. J. Almeida, Lipid nanoparticles containing oryzalin for the treatment of leishmaniasis. *Eur. J. Pharm. Sci.* 45, 442 **(2012)**.
26. A. J. Almeida and E. Souto, Solid lipid nanoparticles as a drug delivery system for peptides and proteins. *Adv. Drug Deliv. Rev.* 59, 478 **(2007)**.

27. E. B. Souto, A. J. Almeida, and R. H. Müller, Lipid Nanoparticles (SLN®, NLC®) for cutaneous drug delivery: Structure, protection and skin effects. *J. Biomed. Nanotechnol.* 3, 317 **(2007)**.

28. S. H. Jung, D. H. Lim, S. H. Jung, J. E. Lee, K.-S. Jeong, H. Seong, and B. C. Shin, Amphotericin B-entrapping lipid nanoparticles and their *in vitro* and *in vivo* characteristics. *Eur. J. Pharm. Sci.* 37, 313 **(2009)**.

29. N. P. Aditya, S. Patankar, B. Madhusudhan, R. S. R. Murthy, and E. B. Souto, Arthemeter-loaded lipid nanoparticles produced by modified thin-film hydration: Pharmacokinetics, toxicological and *in vivo* anti-malarial activity. *Eur. J. Pharm. Sci.* 40, 448 **(2010)**.

30. A. P. Nayak, W. Tiyaboonchai, S. Patankar, B. Madhusudhan, and E. B. Souto, Curcuminoids-loaded lipid nanoparticles: Novel approach towards malaria treatment. *Colloid Surf. B-Biointerfaces* 81, 263 **(2010)**.

31. M. L. Corvo, O. C. Boerman, W. J. Oyen, J. C. Jorge, M. E. Cruz, D. J. Crommelin, and G. Storm, Subcutaneous administration of superoxide dismutase entrapped in long circulating liposomes: *In vivo* fate and therapeutic activity in an inflammation model. *Pharm. Res.* 17, 600 **(2000)**.

32. G. Rouser, S. Fkeischer, and A. Yamamoto, Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5, 494 **(1970)**.

33. J. F. Alzate, A. Alvarez-Barrientos, V. M. González, and A. Jiménez-Ruiz, Heat-induced programmed cell death in *Leishmania infantum* is reverted by Bcl-X(L) expression. *Apoptosis* 11, 161 **(2006)**.

34. M. A. Esteves, I. Fragiadaki, R. Lopes, E. Scoulica, and M. E. M. Cruz, Synthesis and biological evaluation of trifluralin analogues as antileishmanial agents. *Bioorg. Med. Chem.* 18, 274 **(2010)**.

35. D. Plano, Y. Baquedano, D. Moreno-Mateos, M. Font, A. Jiménez-Ruiz, J. A. Palop, and C. Sanmartín, Selenocyanates and diselenides: A new class of potent antileishmanial agents. *Eur. J. Med. Chem.* 46, 3315 **(2011)**.

36. H. Castro, F. Teixeira, S. Romao, M. Santos, T Cruz, M. Flórido, R. Appelberg, P. Oliveira, F. Ferreira-da-Silva, and A. M. Tomás, Leishmania mitochondrial peroxiredoxin plays a crucial peroxidase-unrelated role during infection: Insight into its novel chaperone activity. *PLoS Pathog.* 7, e1002325 **(2011)**.

37. P. A. Buffet, A. Sulahian, Y. J. Garin, N. Nassar, and F. Derouin, Culture microtitration: A sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. *Antimicrob. Agents Chemother.* 39, 2167 **(1995)**.

38. P. Escobar, V. Yardley, and S. L. Croft, Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid mice. *Antimicrob. Agents Chemother.* 45, 1872 **(2001)**.

39. S. Nema, R. J. Brendel, and R. Washkuhn, Encyclopedia of Pharmaceutical Technology, 3rd edn., edited by James Swarbrick, Informa Healthcare USA, Inc., New York **(2007)**, Vol. 1, pp. 1622–1645.

40. T. M. Allen, G. A. Austin, A. Chonn, L. Lin, and K. C. Lee, Uptake of liposomes by cultured mouse bone marrow macrophages: Influence of liposome composition and size. *Biochim. Biophys. Acta* 1061, 56 **(1991)**.

41. T. M. Riddick, Control of Colloid Stability Through Zeta Potential: With a Closing Chapter on Its Relationship to Cardiovascular Disease, Zeta-Meter Inc., Livingston Pub. Co., Wynnewood **(1968)**.

42. M. V. Zhukova, O. V, Romanenko, V. A. Nikolaevich, and M. A. Kisel, Hemolytic properties of miltefosine in liposomes of various lipid compositions. *Pharm. Chem. J.* 44, 507 **(2010)**.

43. G. W. Boswell, D. Buell, and I. Bekersky, AmBisome (liposomal amphotericin B): A comparative review. *J. Clin. Pharmacol.* 38, 583 **(1998)**.

44. M. D. Joshi and R. H. Müller, Lipid nanoparticles for parenteral delivery of actives. *Eur. J. Pharm. Biopharm.* 71, 161 **(2009)**.

45. S. Gupta, A, Pal, and S. P. Vyas, Drug delivery strategies for therapy of visceral leishmaniasis. *Expert Opin. Drug Deliv.* 7, 371 **(2010)**.

46. L. H. Reddy, R. K. Sharma, K. Chuttani, A. K. Mishra, and R. S. R. Murthy, Influence of administration route on tumor uptake and biodistribution of etoposide loaded solid lipid nanoparticles in Dalton's lymphoma tumor bearing mice. *J. Control Release* 105, 185 **(2005)**.

47. T. M. Allen, C. B. Hansen, and L. S. Guo, Subcutaneous administration of liposomes: A comparison with the intravenous and intraperitoneal routes of injection. *Biochim. Biophys. Acta* 1150, 9 **(1993)**.

48. S. Chono, Y. Taushi, and K. Morimoto, Influence of particle size on the distribuitions of liposomes to artherosclerotic lesions in mice. *Drug Dev. Ind. Pharm.* 32, 125 **(2006)**.

49. T. M. Houng, H. Harashima, and H. Kiwada, *In vivo* studies on the role of complement in the clearance of liposomes in rats and guinea pigs. *Biol. Pharm. Bull.* 22, 515 **(1999)**.

50. H. Harashima, T. M. Houng, T. Ishida, Y. Manabe, H. Matsuo, and H. Kiwada, Synergistic effect between size and cholesterol content in enhanced hepatic uptake clearance of liposomes through complement activation in rats. *Pharmaceu. Res.* 13, 1704 **(1996)**.

51. T. M. Allen and C. Hansen, Pharmacokinetics of stealth versus conventional liposomes: Effect of dose. *Biochim. Biophys. Acta* 1068, 133 **(1991)**.

52. R. M. Abra and C. A. Hunt, Liposome disposition *in vivo*. III. Dose and vesicle-size effects. *Biochim. Biophys. Acta* 666, 493 **(1981)**.

53. S. M. Moghimi, A. C. Hunter, and J. C. Murray, Long-circulating and target-specific nanoparticles: Theory to practice. *Pharmacol. Rev.* 53, 283 **(2001)**.

54. S. M. Moghimi, C. J. Porter, I. S. Muir, L. Illum, and S. S. Davis, Non-phagocytic uptake of intravenously injected microspheres in rat spleen: Influence of particle size and hydrophilic coating. *Biochem. Biophys. Res. Commun.* 177, 861 **(1991)**.

55. S. Medda, P. Jaisankar, R. K. Manna, B. Pal, V. S. Giri, and M. K. Basu, Phospholipid microspheres: A novel delivery mode for targeting antileishmanial agent in experimental leishmaniasis. *J. Drug Target* 11, 123 **(2003)**.

56. S. Medda, S. Mukhopadhyay, and M. K. Basu, Evaluation of the *in-vivo* activity and toxicity of amarogentin, an antileishmanial agent, in both liposomal and niosomal forms. *J. Antimicrob. Chemother.* 44, 791 **(1999)**.