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Research Paper

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

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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.

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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