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Research paper

# Formulation of oryzalin (ORZ) liposomes: *In vitro* studies and *in vivo* fate

Rui M. Lopes [a], M. Luísa Corvo [a], Carla V. Eleutério [a], Manuela C. Carvalheiro [a], Effie Scoulica [b], M. Eugénia M. Cruz [a,*]

[a] *iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisboa, Portugal*
[b] *School of Medicine, Laboratory of Clinical Bacteriology, Parasitology, University of Crete, Heraklion, Greece*

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ABSTRACT

Oryzalin (ORZ) is a dinitroaniline that has attracted increasing interest for the treatment of leishmaniasis. The possible use of ORZ as an antiparasitic agent is limited by low water solubility associated with an *in vivo* rapid clearance. The aim of this work was to overcome these unfavorable pharmaceutical limitations potentiating ORZ antileishmanial activity allowing a future clinical use. This was attained by incorporating ORZ in appropriate liposomes that act simultaneously as drug solvent and carrier delivering ORZ to the sites of *Leishmania* infection. The developed ORZ liposomal formulations efficiently incorporated and stabilised ORZ increasing its concentration in aqueous suspensions at least 150 times without the need of toxic solvents. The incorporation of ORZ in liposomes reduced the *in vitro* haemolytic activity and cytotoxicity observed for the free drug, while ORZ exhibits a stable association with liposomes during the first 24 h after parenteral administration, significantly reducing ORZ blood clearance and elimination from the body. Simultaneously, an increased ORZ delivery was observed in the main organs of leishmanial infection with a 9–13-fold higher accumulation as compared to the free ORZ. These results support the idea that ORZ performance was strongly improved by the incorporation in liposomes. Moreover, ORZ liposomal formulations can be administrated *in vivo* in aqueous suspensions without the need of toxic solvents. It is expected an improvement in the therapeutic activity of liposomal ORZ that will be tested in future work.



## 1. Introduction

Leishmaniasis is a disease caused by over 20 different species and subspecies of protozoan parasites of the genus *Leishmania* that affects about 12 million people worldwide [1]. The transmission of this disease occurs through hematophagus vectors and, depending on the causative species, human leishmaniasis can manifest as cutaneous (CL), mucocutaneous (MCL) or visceral (VL) forms, being VL fatal if left untreated [2,3]. Although prevalent in tropical and subtropical regions, leishmaniasis is also endemic to southern Europe [4]. In Europe, South America, Asia and Africa, co-infection with *leishmania* and HIV is becoming an emerging problem and has already been reported in 35 of the 88 VL-endemic countries [5,6].

For more than 70 years, the first-line treatment in most countries has been injectable pentavalent antimonials (Pentostam® and Glucantime®). The treatment is lengthy, potentially toxic and painful; it has become ineffective in parts of India and Nepal as resistance has been developed. Second-line treatments include drugs like diamidinepentamidine, paromomycin and amphotericin B, lipid formulations of amphotericin B (AmBisome®) and the first oral drug miltefosine. However, their use is also limited either due to toxicity and/or long treatment courses, high costs in addition to the emergence of resistance [7]. For these reasons, there is an urgent need of new antileishmanial drugs.

Dinitroanilines like Trifluralin (TFL) and Oryzalin (ORZ) are widely used in herbicide formulations. Their herbicidal effect is due to an antimitotic activity that in turn is determined by the binding of the dinitroanilines to tubulins, the main structural component of microtubules [8]. The interaction of dinitroanilines with tubulins is extremely specific: these substances efficiently bind to tubulins of plant and protozoan and practically do not bind to

*Abbreviations:* CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis; TFL, Trifluralin; ORZ, Oryzalin; DPPC, Dipalmitoylphosphatidylcholine; DPPG, Dipalmitoylphosphatidylglycerol; DMPC, Dimyristoylphosphatidylcholinel; DMPG, Dimyristoylphosphoglycerol; FBS, foetal bovine serum; THP-1, human monocytic cell line; I.E., incorporation efficacy; L.C., loading capacity; Ø, mean diameter; PI, polydispersity index; ζ, zeta potential; TFA, trifluoroacetic acid; RBCs, red blood cells; $T_t$, phase transition temperatures; Lip-$^{14}$C-ORZ, ORZ–liposome associated labelled with $^{14}$C; $^{3}$H-Lip, cholesterol–liposome associated labelled with $^{3}$H; Free-$^{14}$C-ORZ, free ORZ labelled with $^{14}$C; % ID, percentage of injected dose.

* Corresponding author. iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal. Tel.: +351 217500764.
*E-mail address:* eugeniacruz@ff.ul.pt (M.E.M. Cruz).

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animal and fungal tubulins [9]. Due to this specificity, this group of compounds includes promising antiparasitic agents with proven activity against tubulin of several parasitic protozoa, such as *Trypanosoma* spp., *Plasmodium falciparum*, *Toxoplasma gondii* and *Leishmania* spp. [9–12]. Previous studies had already shown that ORZ and TFL are active *in vitro* against *Leishmania tropica*, *Leishmania major*, *Leishmania donovani* and *Leishmania infantum* [10,13,14]. Nevertheless, the use of ORZ (Fig. 1) as an antiparasitic agent may be limited by its low water solubility (*e.g.*, ORZ aqueous solubility of 2.5 mg/L).

This weakness may create difficulties on formulation development: the need to use organic solvents that may lead to undesirable side effects, the heterogeneous results in biological assays, which may be difficult to interpret and the need to administer higher, and possibly toxic dosages for *in vivo* purposes. One of the strategies currently being used to overcome this situation is the incorporation of drugs in liposomes. Liposomes are phospholipid based synthetic vesicles that allow the entrapment of both hydrophilic and hydrophobic drugs. These delivery systems not only enable the safe transport of high drug concentrations into living organisms, but also provide a possible mean of drug targeting to specific cells or organs. In *Leishmania* infections, a specific targeting arises from the observed fact that conventional liposomes are rapidly removed from the circulation by the mononuclear phagocyte system cells (MPS) after intravenous (i.v.) administration [15,16]. The MPS includes macrophages from liver, spleen or bone marrow, which act as obligate host cell systems for *Leishmania* parasites, which are in turn specifically targeted by conventional liposomes.

Since Black et al. [17] first description of the strategic utility of the i.v. administration of liposomal pentavalent antimony, liposome application on the treatment of leishmaniasis has been studied for a wide variety of antileishmanial agents. Liposomes incorporating current antileishmanial drugs (pentavalentantimonials [18], paromomycin [19] and miltefosine [20]) or new agents (atovaquone [21], harmine [22]) have shown improved antileishmanial performance against experimental VL as compared to the free drugs. The advantages of using new formulations are limited due to the existence of resistant strains (*e.g.*, pentavalent antimonials, miltefosine) or due to the early stage of research (*e.g.*, atovaquone, harmine and paromomycin). The commercialised liposomal amphotericin B, AmBisome®, not only improved the drug therapeutic efficacy but also reduced its liver and kidneys toxicity [23]. This formulation is considered the first choice treatment for patients who are unresponsive to antimonials [24]. However, the cost of such treatment is currently too high for the massive use in developing countries [25,26]. In view of the present scenario, it will be interesting to explore the potentialities of alternative antileishmanial agents and to develop appropriate formulations [27].

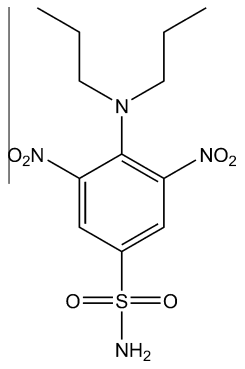


**Fig. 1.** ORZ chemical structure (3,5-dinitro-N′,N′-dipropylsulfanilamide, $C_{12}H_{18}N_4O_6S$). ORZ is a yellow-orange crystalline solid with a molecular weight of 346.36, soluble in water at 2.5 mg/L at pH 7/25 °C and p*Ka* of 8.6.

To the best of our knowledge, there are no studies concerning the development of liposomal formulations for ORZ. However, the successful application of liposomes for dinitroanilines incorporation was already described in the literature for TFL. Liposomal formulations incorporating TFL showed superior activity *in vivo* as compared to the free drug in a murine visceral model of infection (*L. donovani*) [28] as well as in the treatment of experimental canine leishmaniasis (*L. infantum*) [29], while no sterilisation of the parasites was achieved.

The aim of this work is the development of liposomal formulations of ORZ, the evaluation of their *in vitro* behaviour and their pharmacokinetic and biodistribution profile in the perspective of their future application as a new antileishmanial therapy.

## 2. Materials and methods

### 2.1. Materials

ORZ was purchased from Supelco (Bellefonte, USA), and pure phospholipids (Dipalmitoylphosphatidylcholine – DPPC; Dipalmitoylphosphatidylglycerol – DPPG; Dimyristoylphosphatidylcholine – DMPC and Dimyristoylphosphoglycerol – DMPG) were supplied by Avanti Polar Lipids, Inc. (Alabaster, USA). Radiolabeled [$^{14}$C] ORZ (4-aminosulfonyl-2,6-dinitro-N,N-di-n-propylaniline-Ph-UL-$^{14}$C, specific activity: 6.5 mCi/mmol, uniformly labelled on the aromatic ring) was an offer from Dow AgroSciences (Indianapolis, USA). Radiolabeled [$^{3}$H]Chol ([1a,2a(n)-3H]Cholesterol, specific activity: $44 \times 10^3$ mCi/mmol) was obtained from Amersham Radiochemicals (Amersham, UK). Acetonitrile (HPLC grade) was from Merck. RPMI 1640 media (20 mM HEPES), penicillin–streptomycin and foetal bovine serum (FBS) were purchased from Sigma–Aldrich (USA). LIVE/DEAD viability kit was obtained from Molecular Probes (UK). All other reagents were analytical grade.

### 2.2. Cell lines and animals

The human monocytic cell line THP-1 was maintained in culture 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% $CO_2$. Promastigotes of *L. infantum* MHOM/TN/80/IPT1/LEM 235 were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine and antibiotics, at 26 °C.

$CD_1$ male mice weighting 25–30 g were obtained from Charles River, Barcelona, Spain. Animals were fed with standard laboratory food and water *ad libitum*. All animal experiments were carried out with the permission of 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 I-A 67/92, and all following legislations.

### 2.3. Preparation of ORZ liposomal formulations

The incorporation of ORZ in liposomes was performed by the dehydration-rehydration method (DRV) [30,31] with some modifications. Briefly, the appropriate amounts of phospholipids (10 mM) and ORZ (86.5–865 μg/mL) 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 Moduyo freeze-dryer (Edwards, Germany). The lyophilised powder was rehydrated in two steps: first with a trehalose–citrate buffer (10 mM citrate, 135 mM NaCl, 30 mM trehalose) pH 5.5 (2/10 of the final volume) followed by mild vortexing and left at room temperature for 30 min. The hydration was completed with the addition of the remaining volume (8/10 of the final volume) of