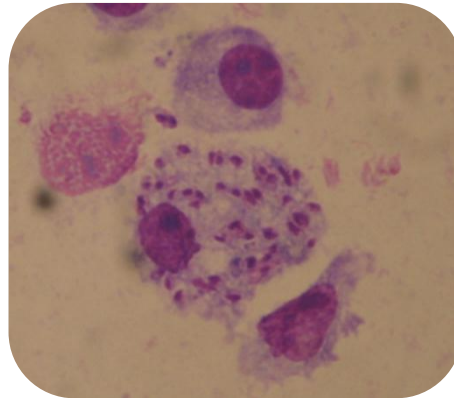


Oryzalin liposomal formulations as antileishmanial agents: Characterization, *in vitro* and *in vivo* evaluation



Lopes R, Carvalheiro M, Eleutério CV, Corvo ML, Almeida AJ, Cruz MEM

iMed.UL- Research Institute for Medicines and Pharmaceutical
Sciences, Faculdade de Farmácia, Universidade Lisboa

**Workshop Antiparasitic and Antitumor drugs,
8 and 9 of September 2011, IBMC, Porto, Portugal**



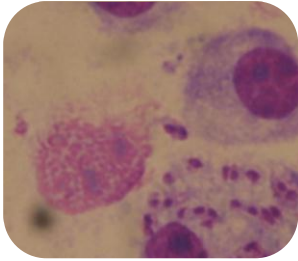
Currently there is exists **no effective vaccine** against Leishmaniasis and chemotherapy remains to be the only option.



Current therapeutic options limited by toxicity, resistance, long course or high cost.

New Drugs

- Alkylphosphocholines
- Arylimidamides
- Bisphosphonates
- Dinitroanilines
- Pyrazinamide
- Quinolines
-

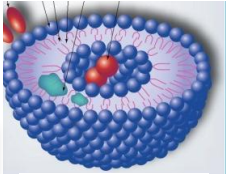


Target

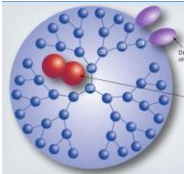
- Liver
- Spleen

New Strategies

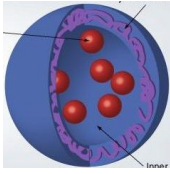
Drug Delivery Systems



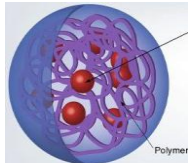
Liposomes



Dendrimers



Nanocapsules

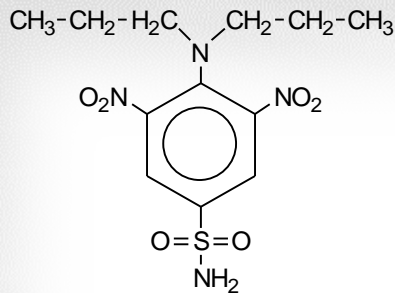


Nanospheres



Solid Lipid Nanoparticles

- ORZ is a dinitroaniline widely used in agricultural practice as an herbicide.



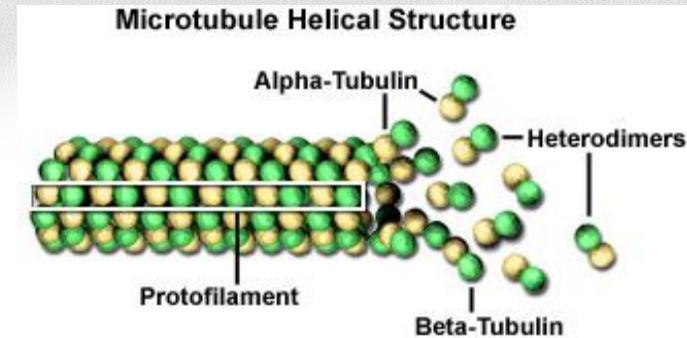
Oryzalin (ORZ)

Specific Binding to Tubulin



Plant
Protozoan

Animal



In vitro Activity

Trypanosoma cruzi,

Cryptosporidium parvum

Toxoplasma gondii

Plasmodium falciparum

L. major, *L. tropica* (cutaneous)

L. donovani, *L. infantum* (visceral)

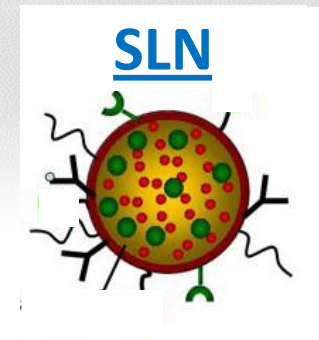
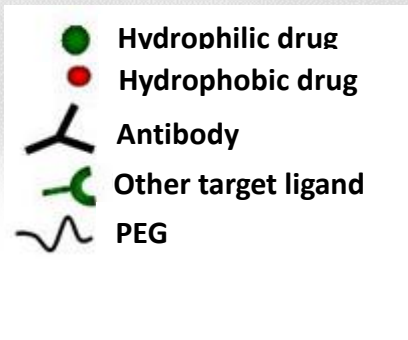
L. pananensis (mucocutaneous)

Drawbacks

Water solubility
(mg/L) at 25 °C

2.5 @ pH= 7

Dinitroaniline therapeutic use is limited at therapeutic doses, in a vehicle suitable for i.v. administration.



Lipid vesicles made from a phospholipids bilayer surrounding a aqueous core.

Lipid particles made from a solid lipid core stabilized by a surfactant interfacial region.

Used as drug carriers and loaded with a great variety of molecules:

small drug molecules, proteins, nucleotides and even plasmids.

Control and/or target drug release.

Improved stability of pharmaceuticals.

Excellent biocompatibility.

Objective: Perform a systematic and comparative study of 2 drug delivery systems of lipidic nature (Liposomes and Solid Lipid Nanoparticles) for improvement of ORZ performance

- **Construction of Liposome as delivery systems for the incorporation of ORZ**

- **Stability studies of ORZ liposomal formulations**
 - In suspension
 - freeze-dried and
 - sterilization by autoclaving

Phospholipids: Dimyristoyl phosphatidylcholine (**DMPC**), Dimyristoyl phosphatidylglycerol (**DMPG**)
Dipalmitoyl phosphatidylcholine (**DPPC**), Dipalmitoyl phosphatidylglycerol (**DPPG**)

Preparation Method: Dehydration–Rehydration Vesicles (**DRV**)



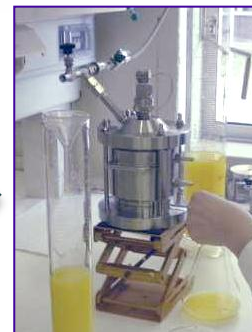
Lipidic Film



Hydration of lipid film



Freeze-drying followed by re-hydration



Extrusion



Liposome suspension

Formulation	L.C ($\mu\text{g}/\mu\text{mol}$)	I.E. (%)	ORZ Yield (%)	\emptyset (nm)	ζ (mV)
DMPC:DMPG (7:3)	29 \pm 3	88 \pm 4	77 \pm 4	142 \pm 21	-41 \pm 3

ORZ Water solubility:
0.0025 mg/mL

1250x increase

ORZ Liposomal Concentration:
3.125 mg/mL

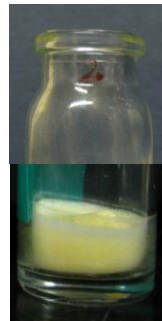
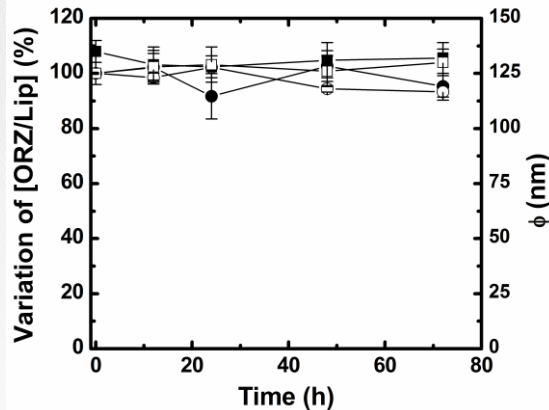
DMPC:DMPG:ORZ (7:3:1)
Liposome ϕ : 155 \pm 21 nm
PdI: 0.15



In suspension
(at room temperature)

Freeze dried
(Trehalose 30 mM)

Sterilised by autoclaving
(121 °C/15 min)



Liposome ϕ : 155 \pm 30 nm
PdI: 0.25
ORZ retention: 85 \pm 5%



Liposome ϕ : 145 \pm 5 nm
PdI: 0.15
ORZ retention: 90 \pm 3%

Liposomal formulation was **pharmaceutically stable** in suspension at room temperature and **may be lyophilised or autoclaved** without significant variations on its physicochemical proprieties or significant reduction in ORZ incorporation



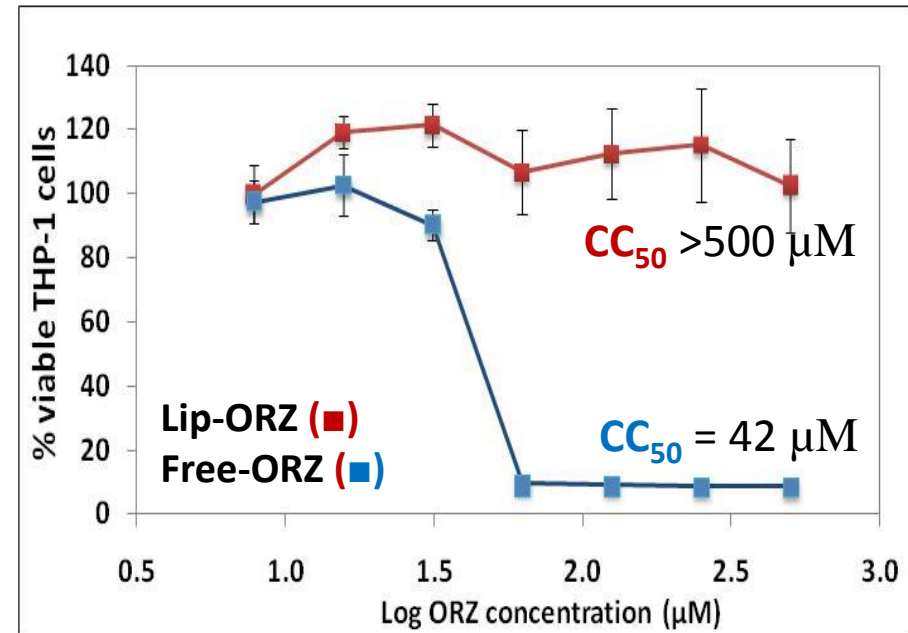
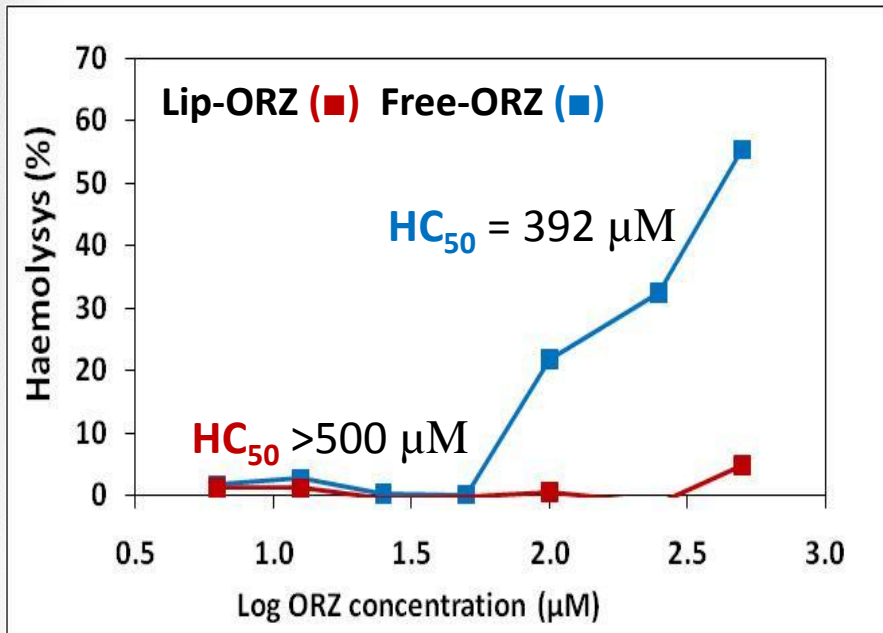
***In vitro* evaluation**

- Hemolysis
- Cytotoxicity (THP-1 cells)
- Internalization assays
- Intracellular activity

Haemolysis : Human red blood cells
Incubation: 37 °C, 1h

Formulation	
ORZ in Tween80 5% (v/v)	Free-ORZ
DMPC:DMPG:ORZ (7:3:1)	Lip-ORZ

Cytotoxicity: THP-1 cell line (MTS method)
Incubation: 37 °C, 72h



- Free-ORZ had a high cytotoxicity and haemolytic activity (HC₅₀ of 392 µM).
- When incorporated in liposomes, no haemolysis were observed at concentrations up to 500 µM.

Formulation

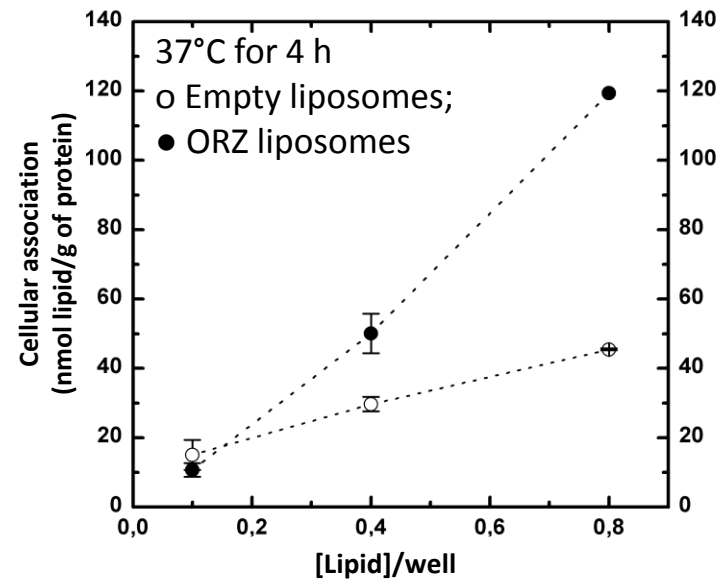
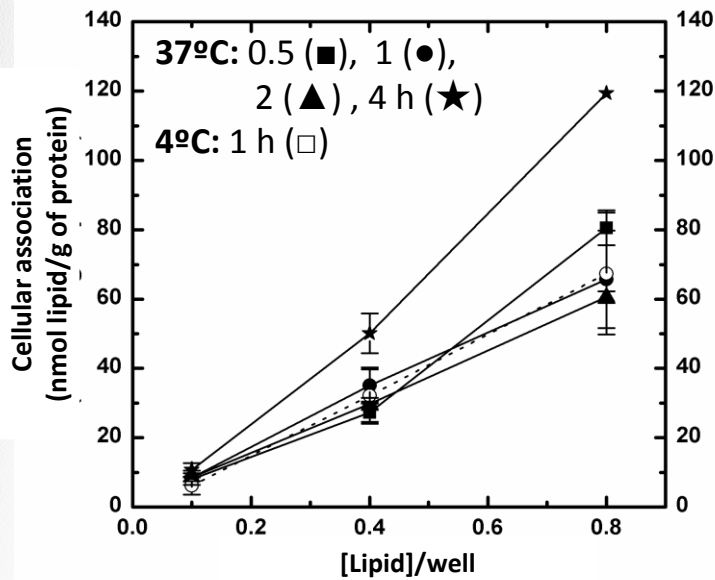
DMPC:DMPG:ORZ (7:3:1)



Rhodamine (Rhod)

Incorporated in liposome bilayer

1×10^6 differentiated THP-1 cells
0.1, 0.4 and 0.8 mM lipid/well
Incubation: 0.5, 1, 2 and 4h (37°C),
1h (4°C)



- Presence of ORZ in the liposome bilayer increases the cellular association after 4h incubation.
- Significant increase ($p < 0.05$) in cellular association at 37 °C (4h incubation).

Formulation

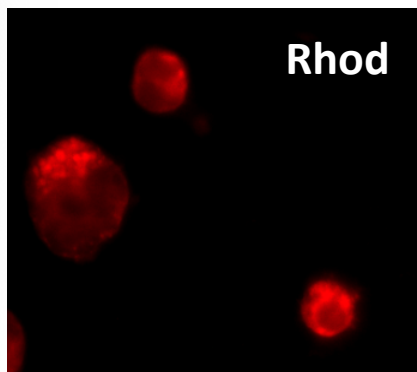
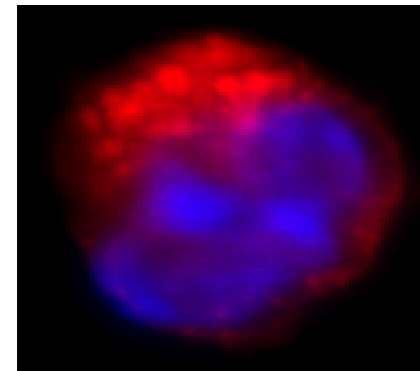
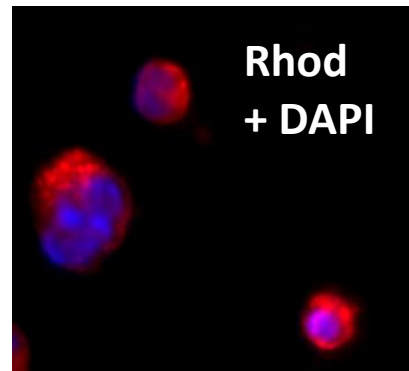
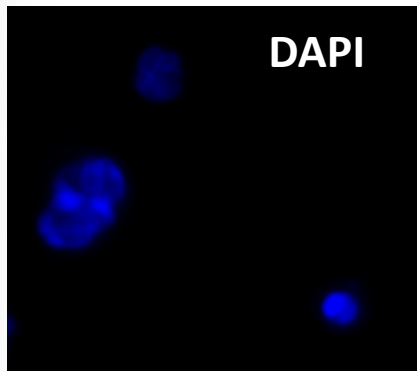
DMPC:DMPG:ORZ (7:3:1)

Rhodamine (Rhod)

Incorporated in
liposome bilayer

DAPI

(4',6-diamidino-2-phenylindole)
Binds to DNA (nucleus)



- The uptake and intracellular localization of rhodamine labeled liposomes was confirmed by fluorescence microscopy.
- Fluorescence was found in the cell cytoplasm as red fluorescent spots.

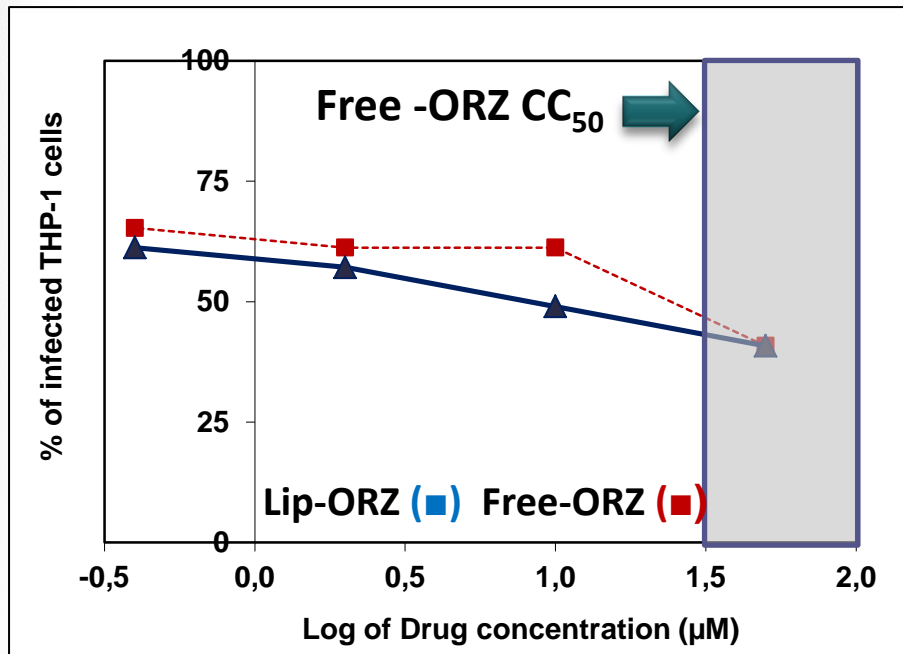
Formulation

ORZ in Tween80 5% (v/v)	Free-ORZ
DMPC:DMPG:ORZ (7:3:1)	Lip-ORZ

THP-1 cell line infected with *L.infantum*

Incubation with Lip-ORZ and Free-ORZ

Count of infected cells in 100 cells



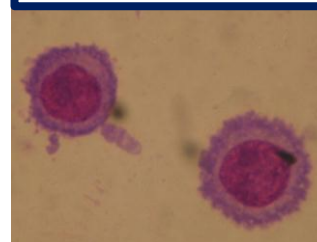
□ Both Free-ORZ and Lip-ORZ reduced the number of THP1 infected cells.

□ Only LIP-ORZ was active at non toxic concentrations

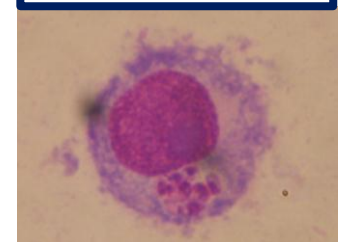
$IC_{50} = 24.3 \mu M$ **Free-ORZ**

$IC_{50} = 8.2 \mu M$ **Lip-ORZ**

Normal THP-1



Infected THP-1



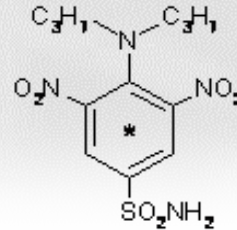


***In vivo* evaluation**

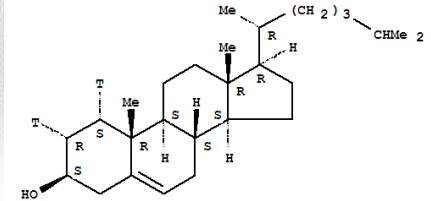
- Blood profile and biodistribution
- Therapeutic activity in *in vivo* animal model

Formulation	Ø (nm)	ζ (mV)
ORZ in Tween80 5% (v/v)		
DMPC:DMPG :ORZ (7:3:1)	142±21	-41±3

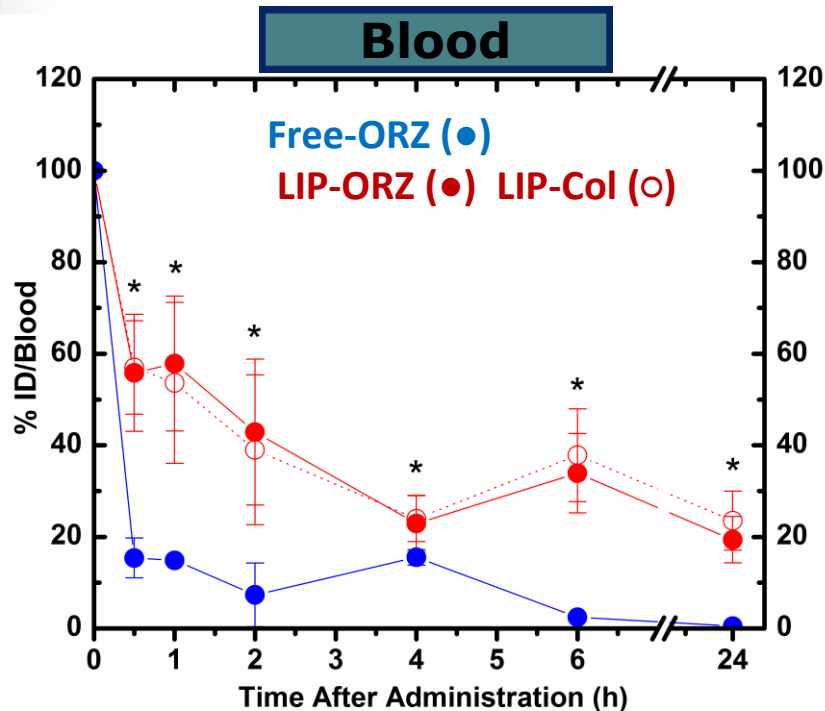
Injection volume: 0.2 mL (i.v);
ORZ dose: 0.15 µmol



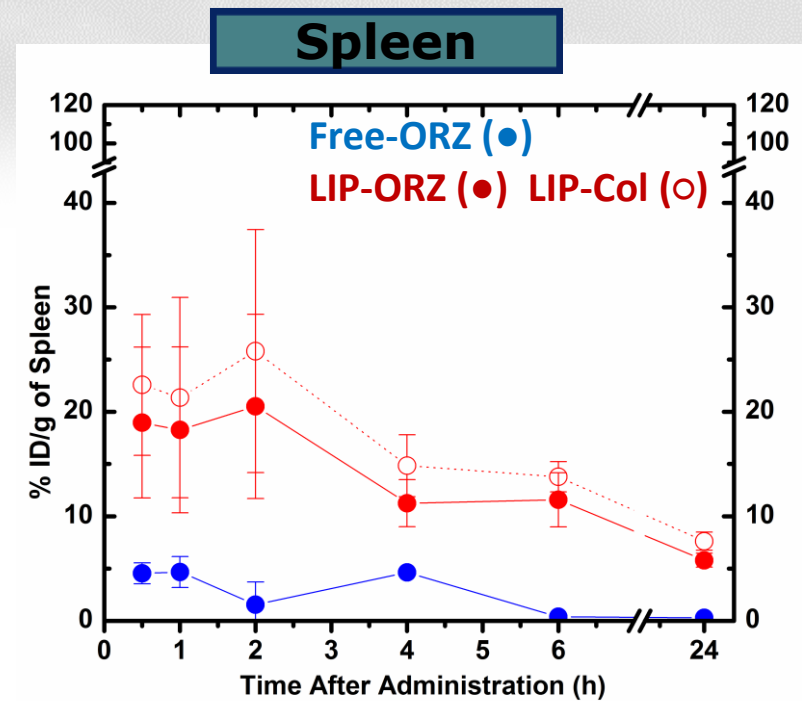
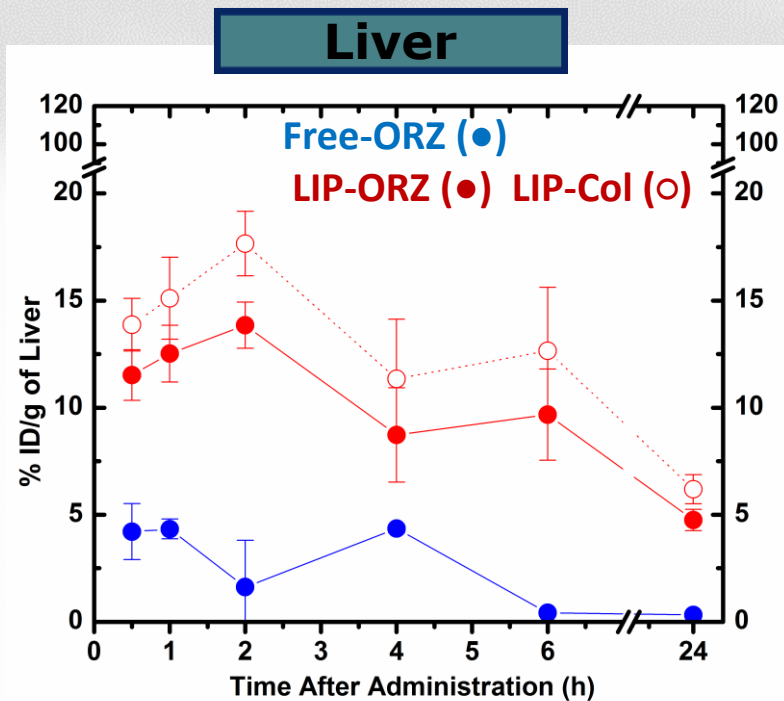
*ORZ uniformly labelled with ¹⁴C on the aromatic ring.



([1a,2a(n)-3H]Cholesterol



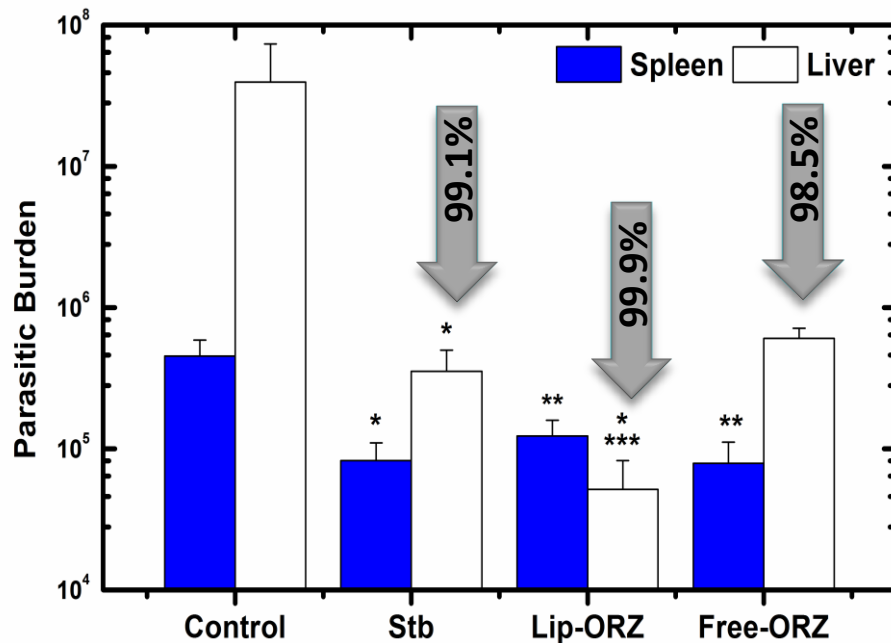
- Rapid decrease/clearance in **Free-ORZ** blood levels after administration
- After 6-24 h only residual amounts of **Free-ORZ** observed



- LIP-ORZ showed a **3 and 4 fold higher accumulation in liver and spleen**, respectively, as compared with the Free-ORZ
- Maximum accumulation observed 2 h post-administration



Treatment		Dose (Administration route)
Glucantime®	Stb	45 mg/kg (s.c.)
DMPC:DMPG:ORZ (7:3:1)	Lip-ORZ	25 mg/kg (i.v.)
ORZ in Tween80 5% (v/v)	Free-ORZ	25 mg/kg (i.v.)



□ All treatments significantly reduced parasitic burden in the liver and spleen.

□ In the liver LIP-ORZ was more active than the Free-ORZ and Stb.

- Liposomal formulations containing ORZ **were optimized** (preparation method, lipid composition and experimental conditions).
- These liposomal formulations are **stable** in different storage conditions (suspension, freeze-dried and sterilized by autoclaving)
- ORZ incorporation in liposomes proved to **reduce haemolysis** of red blood cells and **cytotoxicity** in THP1 cells observed with free ORZ while **improving** its intracellular activity.
- *In vivo* studies demonstrated the efficacy of the liposomal formulation to **target ORZ** to the main organ of leishmanial infections (liver and spleen).
- ORZ incorporation **improved** the *in vivo* activity (visceral model of infection) by reducing the parasitic burden in the **liver and spleen**.

ORZ liposomes are promising formulations as therapeutic agents against leishmaniasis

Further studies include search of new treatment schedules and comparison with similar ORZ Solid lipid nanoparticles formulations.

Faculdade de Farmácia, Universidade de Lisboa (Departamento de Tecnologia Farmacêutica)

Eugénia Cruz

António J. Almeida

Manuela Carvalheiro

Luísa Corvo

Manuela Gaspar

Carla Eleutério



**iMed.UL-Research Institute
for Medicines and
Pharmaceutical Sciences**

School of Medicine, University of Crete

Effie Scoulica – *In vitro* studies



Centro de Neurociências e Biologia Celular (CNC), Universidade de Coimbra



Laboratório Nacional de Energia e Geologia

Alexandra Esteves



Fundação para a Ciência e a Tecnologia

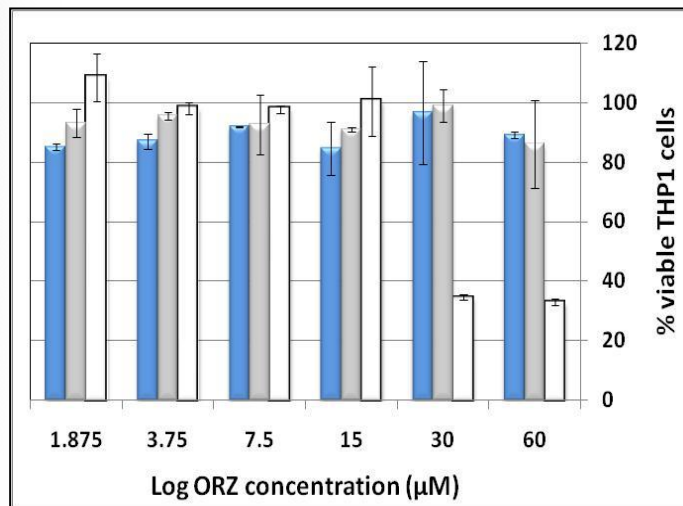
This work was partially supported by FCT (PTDC/CVT/098290/2008). Rui Lopes was recipient of a FCT grant (SFRH/BD/44218/2008)

□ SLN formulations with tripalmitin as the lipidic component were developed

□ **Stability studies of ORZ liposomal formulations**

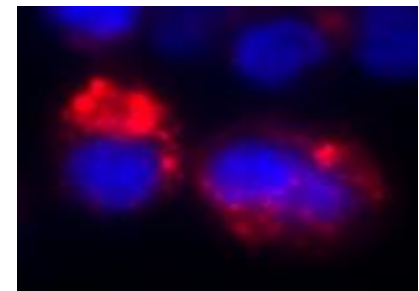
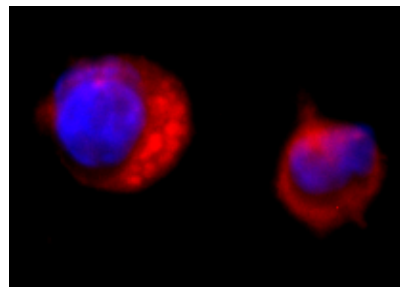
- In suspension
- freeze-dried and
- sterilization by autoclaving

□ Cell viability studies demonstrated that the incorporation of ORZ in SLN decreases the drug cytotoxicity

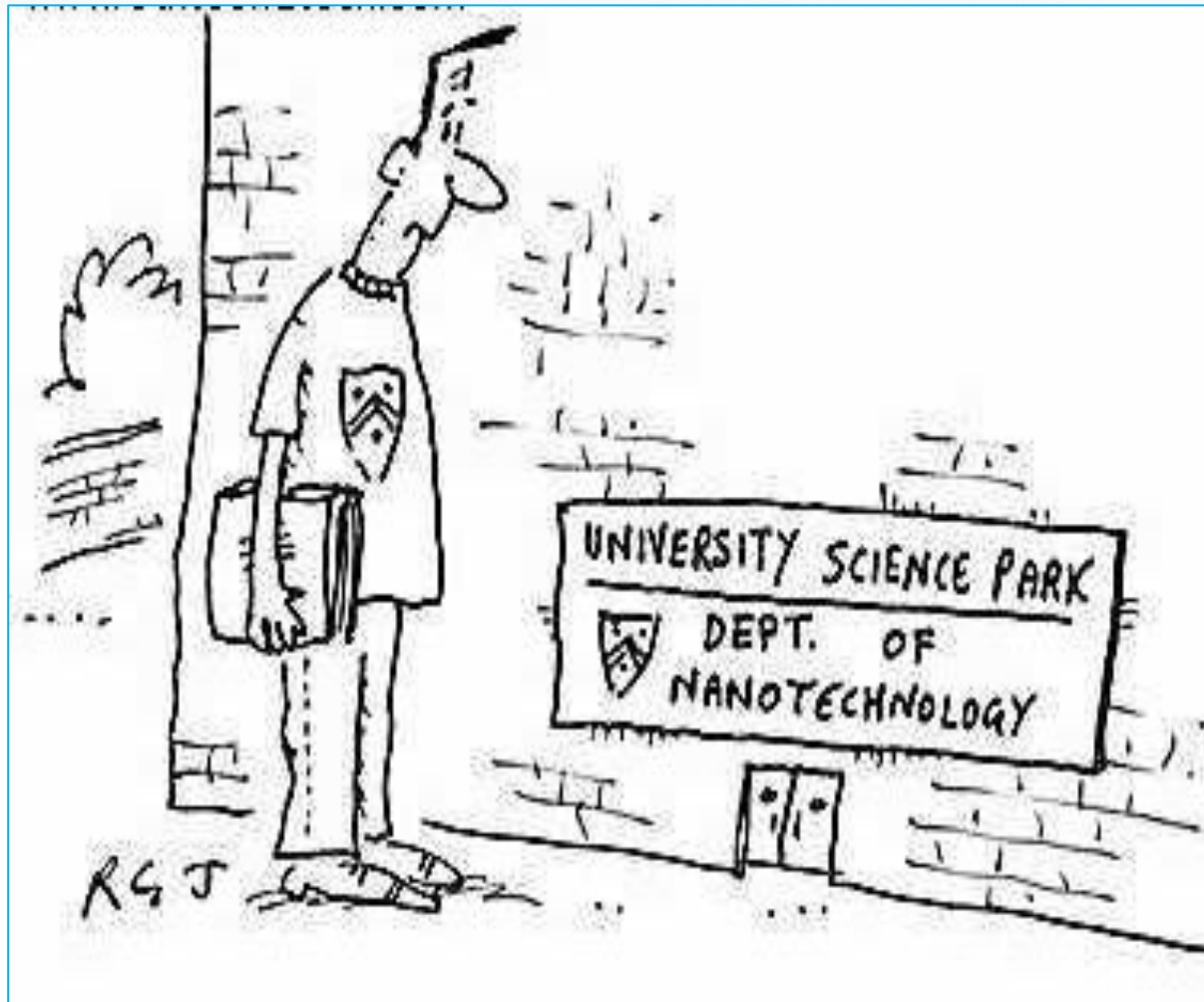


LNP2-ORZ (■), LNP4-ORZ (■), Free ORZ (□).

□ The uptake and intracellular localization of rhodamine labeled SLN was confirmed by fluorescence microscopy.



Thank you for your attention



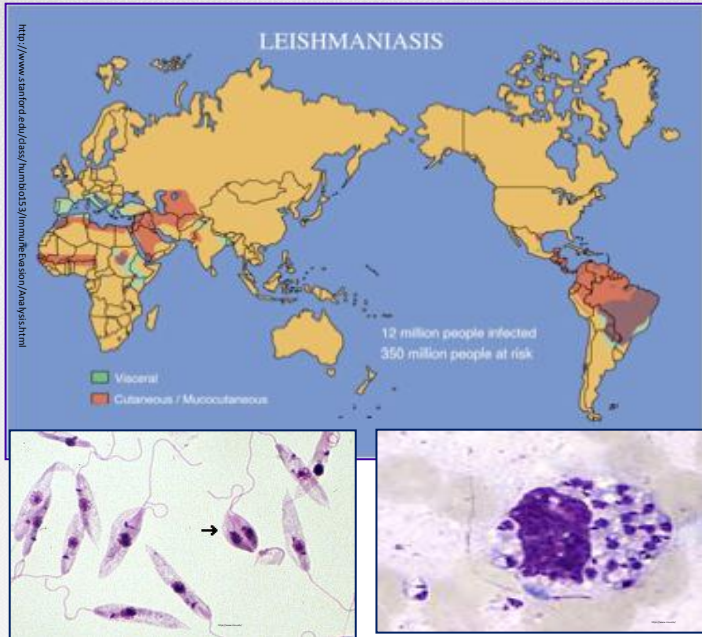
Various liposomal formulations developed against Leishmaniasis – Current treatments

<u>Drug Entrapped</u>	<u>Animal Model</u>	<u>Reference</u>
Meglumine antimoniate and Sodium stibogluconate	<i>L.donovani</i> (golden hamsters)	Alving <i>et al.</i> , (1978)
Meglumine antimoniate	<i>L.donovani</i> (dogs)	Chapman <i>et al.</i> , (1984)
Amphotericin B	<i>L.donovani</i> (hamsters)	Berman <i>et al.</i> (1986)
Amphotericin B	<i>L.infantum</i> (naturally infected dogs)	Oliva <i>et al.</i> , (1995)
Miltefosine	<i>L.donovani</i>	Escobar <i>et al.</i> , (2001)
Meglumine antimoniate	<i>L.Chagasi</i> (naturally infected dogs)	Ribeiro <i>et al.</i> , (2008)
Amphotericin B	<i>L.donovani</i> (BALB/C mice)	Burerjee <i>et al.</i> , (2008)

Various liposomal formulations developed against Leishmaniasis – New drugs

<u>Drug Entrapped</u>	<u>Animal Model</u>	<u>Reference</u>
Atovaquone	<i>L.infantum</i> (mice)	Cauchetier <i>et al.</i> , 2000
Camptothecin	<i>L.donovani</i> (Balb/C mice)	Proulx <i>et al.</i> , 2001
Piperine	<i>L.donovani</i>	Raay <i>et al.</i> , 1999
Trifluralin	<i>L.infantum</i> (dogs)	Marques <i>et al.</i> , 2008
Trifluralin	<i>L.donovani</i> (Balb/C mice)	Carvalho <i>et al.</i> , 2009

- Leishmaniasis is a parasitic disease classified as Neglected Diseases by WHO*.



Leishmania promastigotes

Macrophage infected with Leishmania amastigotes

- The protozoa are transmitted by the bite of the *phlebotomine* sandfly.



- Leishmaniasis presents in three clinical forms:

Cutaneous, Mucocutaneous and Visceral



- Leishmaniasis present in 88 countries (**12 millions infected worldwide**).
- Endemic in all southern countries of Europe.

Currently there is exists **no effective vaccine** against Leishmaniasis and chemotherapy remains to be the only option.

First Line Treatment

Pentavalent Antimonials

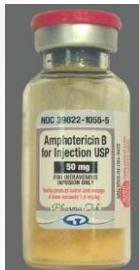
Meglumine Antimoniate (Glucantime)
Sodium stibogluconate (Pentostam)

**Toxicity, Long course treatments;
Painful injection; Resistance**



Second-line treatment

Amphotericin B



**Intravenous infusion
Dose-limiting toxicity**

Paromomycin



**Raised liver enzymes,
Toxicity
Injection site pain**

Miltefosine



**Teratogenicity;
Long half life (potential for
resistance)
Toxicity,**

Liposomal Amphotericin B



High cost of treatments

Current therapeutic options limited by toxicity issues and the need of long course treatments.

<u>Drug Delivery System</u>	<u>Drug Entrapped</u>	<u>Animal Model</u>	<u>Reference</u>
Niosomes	Amphotericin B	<i>L. donovani</i> (BALB/c mice)	Mullen et al., (1997)
Emulsions	Piperine	<i>L. donovani</i> (BALB/c mice)	Veerareddy et al., (2004)
Polymeric Particles	Amphotericin B	<i>L. infantum</i> (hamsters)	Sanchez-Brunete et al., (2004)
Liposomes	Miltefosine	<i>L. donovani</i>	Escobar et al., (2001)
	Meglumine antimoniate	<i>L. Chagasi</i> (naturally infected dogs)	Ribeiro et al., (2008)
	Amphotericin B	<i>L. donovani</i> (BALB/C mice)	Burerjee et al., (2008)
	Atovaquone	<i>L. infantum</i> (mice)	Cauchetier et al., 2000
	Trifluralin	<i>L. donovani</i> (Balb/C mice)	Carvalho et al., 2009