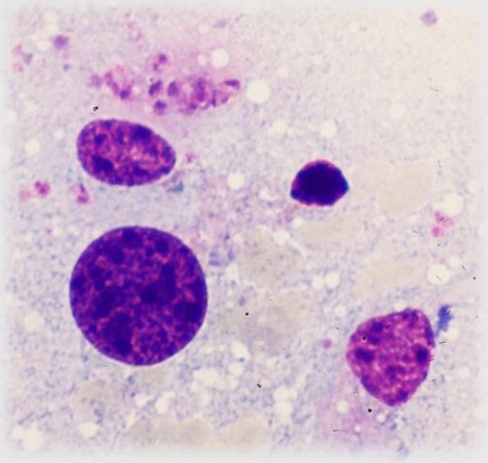
NOVEL DRUGS AND NON-CONVENTIONAL FORMULATIONS AS TOOLS FOR THE TREATMENT OF LEISHMANIA INFECTIONS



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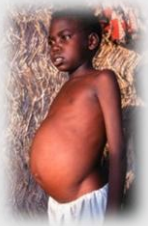
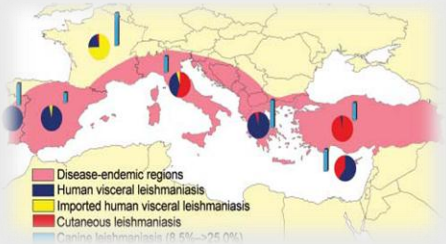
Neglected Tropical Diseases: Leishmaniasis





Leishmania in southern Europe. (Duiardin, 2008)

- Endemic in all southern European countries.
- Most of the reported cases are due to zoonotic VL;
- The dog is a reservoir of the disease;
- Transmitted from reservoirs to humans by female sand flies;

NO VACCINE AVAILABLE

Leishmaniasis: Chemotherapy

Drawbacks

- Low levels at site of infection
  - ↳ Degradation before reaching target tissues
  - ↳ Low drug penetration through infected tissues
- Toxic effects (intolerance, organ damage)
- Long course treatment
- Acquired resistance

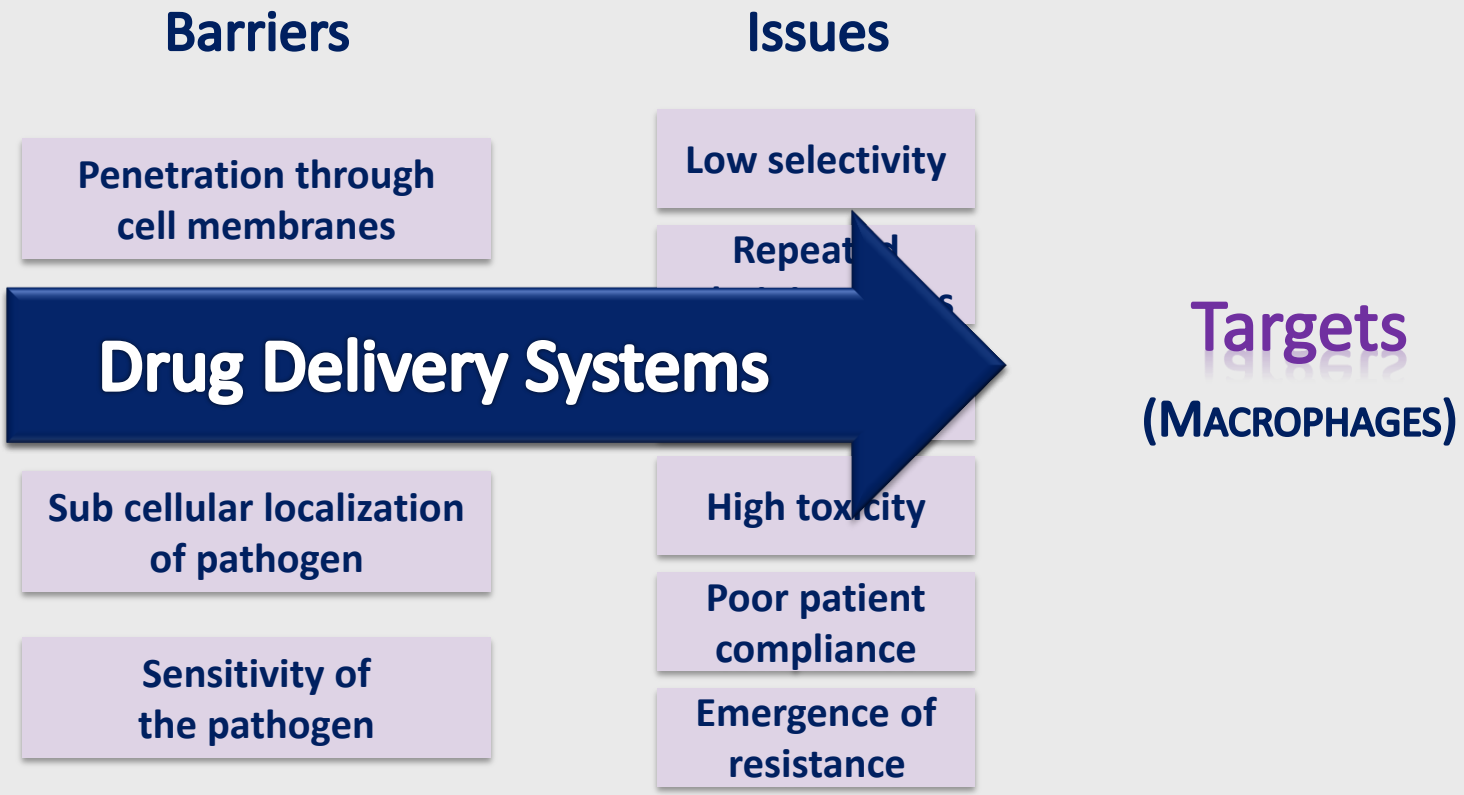
New Approaches

- Search for new antileishmanial compounds
  - ↳ Search for new active molecules
  - ↳ Chemical modification of existing molecules
- Target drugs to the site of infection
  - ↳ Association to drug delivery systems

New Strategies: Macrophage Targeting

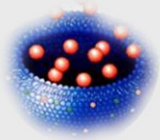
Leishmaniasis

DRUGS have to overcome major structural barriers to reach ***Leishmania*** parasites that live inside mammalian **MACROPHAGES** in different anatomical areas of the host

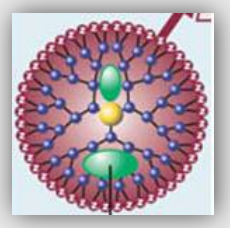


Kayser et al., Parasitol Res (2003) 90: S63–S70

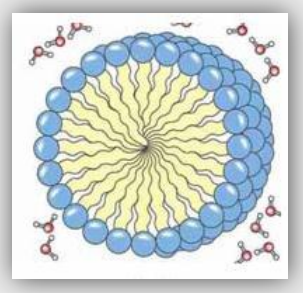
New Strategies: Drug Delivery Systems



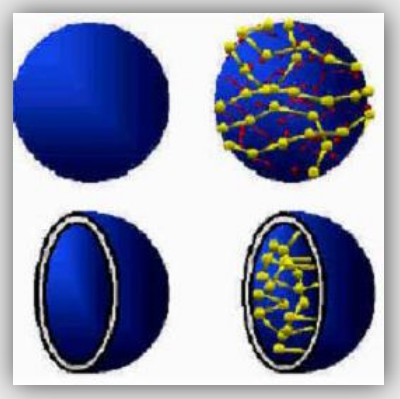
The main goal of a **Drug Delivery System** is to carry the **Bioactive Agent** specifically and safely from the site of administration to the desired therapeutic target in a controlled manner.



Dendrimers



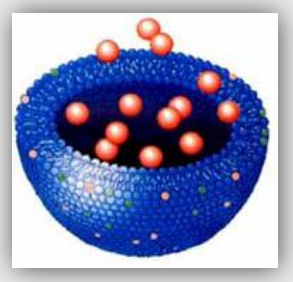
Nanoemulsions



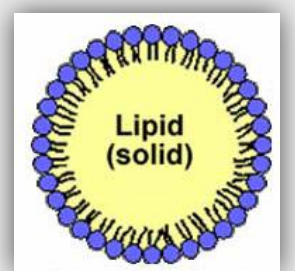
Nanospheres

Nanocapsules

Polymeric Nanoparticles



Liposomes

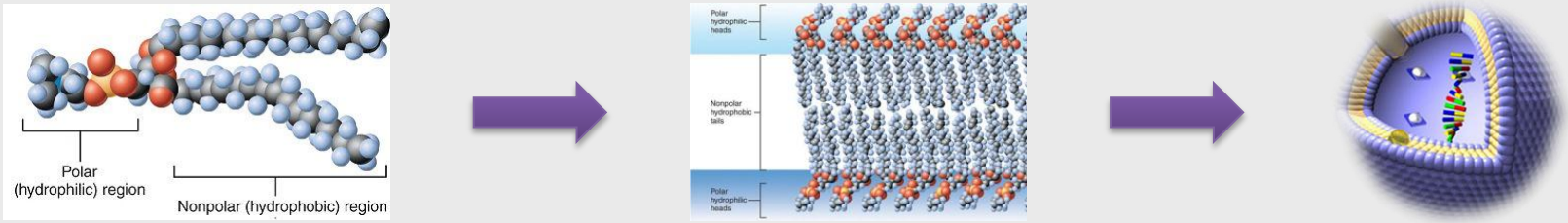


Solid Lipid Nanoparticles

New Strategies: Liposomes as Drug Delivery Systems

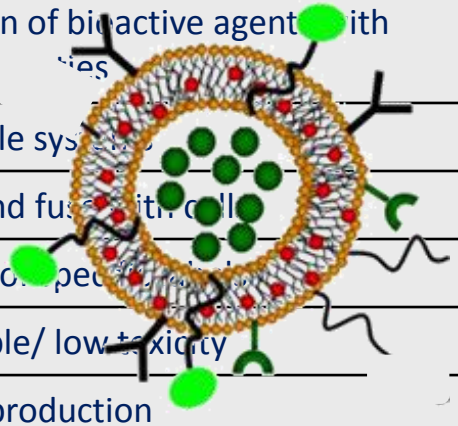
Vesicular concentric bilayer structures mainly made of phospholipids that present a **hydrophilic (polar) head** and **hydrophobic (non polar) fatty acid tail.**

Biocompatible; Biodegradable; Non immunogenic.

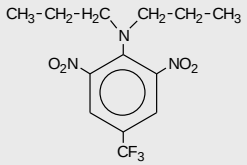


Drug carriers for a great variety of molecules: small drug molecules, proteins, nucleotides and plasmids

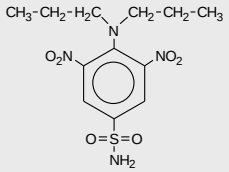
LIPOSOMES PROPERTIES	RATIONAL FOR INCORPORATION
Incorporation of bioactive agents with different properties	**Solubilization** : Hydrophobic Bioactive Agent **Protection** : Hydrophilic Bioactive Agent
Highly flexible system	Tailor made systems
Penetrate and fuse with cells	Cellular internalization of the bioactive agent
Attachment of specific moieties	Change the biodistribution
Biodegradable/ low toxicity	Pharmaceutically acceptable
Large scale production	Pharmaceutically acceptable
Long term stability (2 years)	Pharmaceutically acceptable



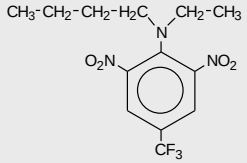
New Strategies: Dinitroanilines



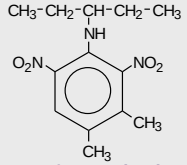
Trifluralin



Oryzalin



Benfluralin



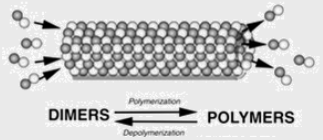
Pendimethalin

Herbicides: Specifically bind to plant and parasites tubulins

Non Toxic: Do not bind to mammalian tubulins

Specific binding to *Leishmania* tubulins causes

- **Microtubule Depolymerisation**
- **Inhibition of Assembly**



- ↳ Inhibition of promastigote proliferation;
- ↳ Decrease promastigote-to-amastigote transformation;
- ↳ Interference with amastigote replication;
- ↳ Reduction in infectivity of amastigotes.

In vitro Activity

Dinitroaniline	IC50 (µM) *L. infantum*	
	Promastigotes	*Amastigotes*
Trifluralin	34,2	19,1
Oryzalin	11,1	11,8
Benfluralin	26,2	17,7
Pendimethalin	23,3	12,6

Drawbacks

Dinitroanilines therapeutic use is limited

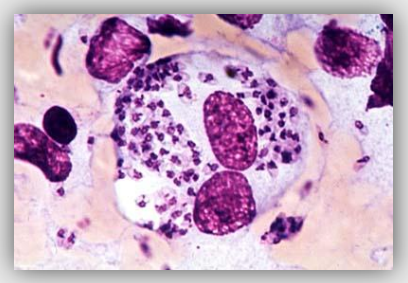
- **Aqueous solubility**
- **Vapour pressure**

Our Strategy: Macrophage Targeted Drug Delivery System

Therapy Targets

Macrophages infected with ***Leishmania*** parasites

(Liver; Spleen; Bone Marrow)



Drugs

Dinitroanilines

↳Commercial (TFL)

↳Chemically modified (TFL-D)

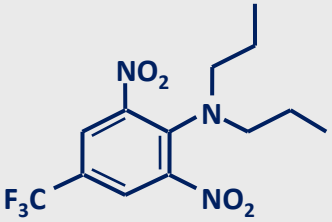
Drug Delivery Systems

Liposomes

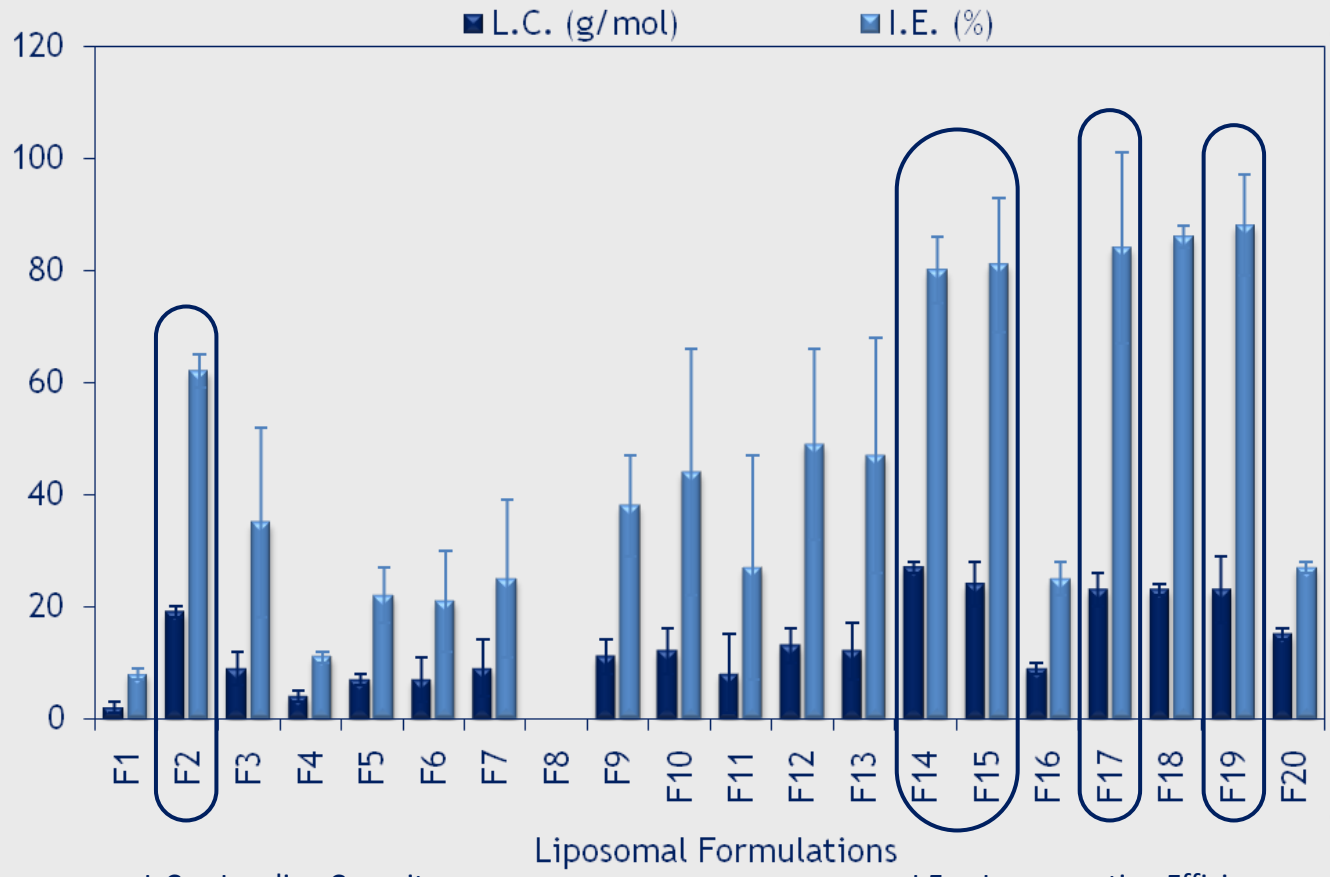
↳Conventional (capture by the MPS)

↳Long Circulating (extravasate vasculature)

Liposomal Trifluralin



Trifluralin (TFL) Formulate **TFL** for systemic application without the need of toxic solvents and at therapeutic doses of cutaneous Leishmaniasis when applied topically as an ointment (Chan *et al.*, 1993)



F1 - PC
F2 - PC:Chol (4:1)
F3 - PC:Chol (2:1)
F4 - DMPC
F5 - DMPC:Chol (4:1)
F6 - DMPC:Chol (2:1)
F7 - DPPC:Chol (4:1)
F8 - DPPC:Chol (2:1)
F9 - DSPC:Chol (4:1)
F10 - DSPC:Chol (2:1)
F11 - PC:Chol:SA (10:5:1)
F12 - PC:Chol:PI (10:5:1)
F13 - PC:Chol:PG (10:5:1)
F14 - PC:PG (7:3)
F15 - DOPC:DOPG (7:3)
F16 - DPPC:DPPG (7:3)
F17 - PC:PG:DSPE-PEG (12:3:1)
F18 - PG:DSPE-PEG (15:1)
F19 - PC:DSPE-PEG (15:1)
F20 - PC:Chol:DSPE-PEG (12:3:1)

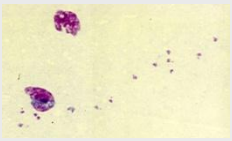
L.C. = Loading Capacity

I.E. = Incorporation Efficiency

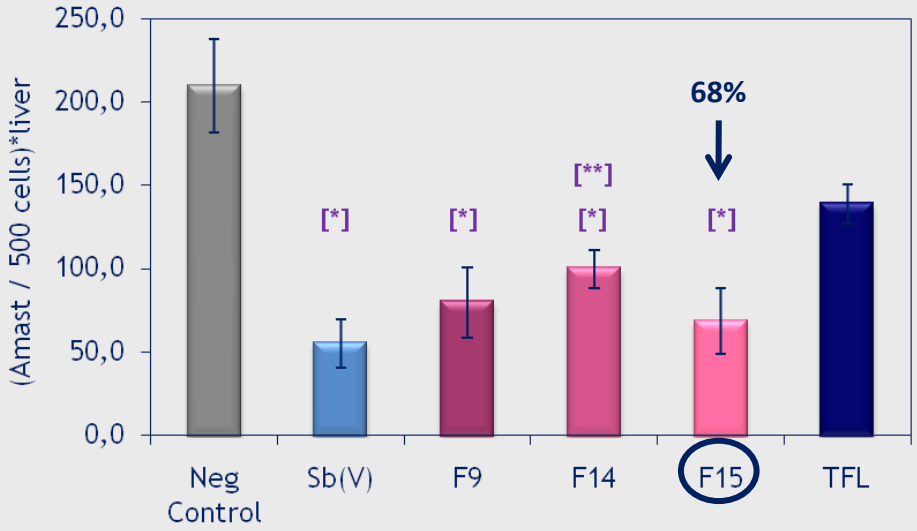
Therapeutic Activity of Liposomal TFL

Visceral Leishmaniasis

L. donovani **MHOM/ET/67/L82** → Infection: $2x10^7$ amast/mouse → **Female BALB/c** → Treatment: 5x i.v.; 15 mg TFL/kg/day; 1 s.c.; 15mg Sbv /kg → **Liver** → Amastigote counting



Effect of the lipid composition



F9 = DSPC:Chol

F14 = PC:PG

F15 = DOPC:DOPG

Liposomal TFL reduces parasite load

[*]-Significantly different from Neg Control and TFL

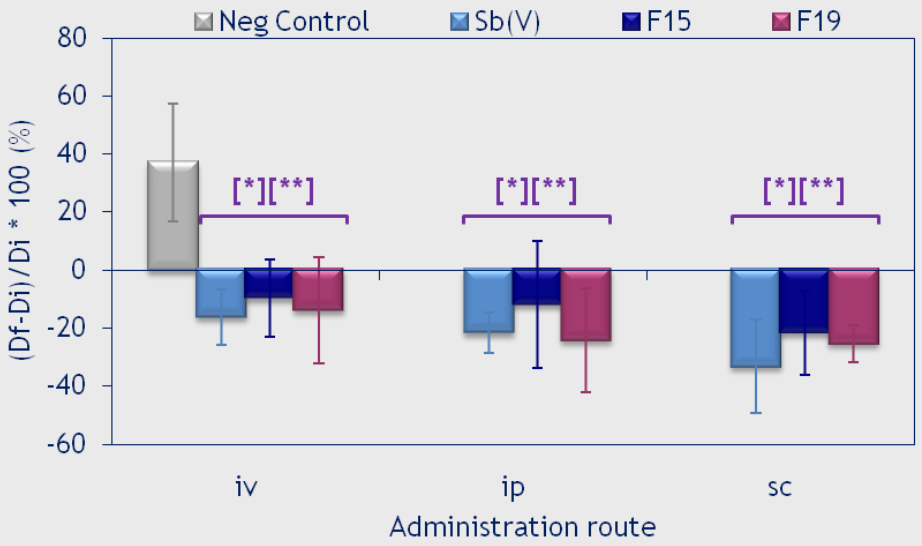
[**]-Significantly different from Sb^V

Therapeutic Activity of Liposomal TFL

Cutaneous Leishmaniasis

L. major **MHOM/SA/85/JISH 118** → Infection: 2 X 10^7 prom/mouse → **Female BALB/c** → 7 days → **Nodule sizing** → Treatment: 6 doses → **Nodule sizing**

Effect of lipid composition and administration route



F15 = DOPC:DOPG

F19 = PC:DSPE-PEG

Sb^v = 400 mg/kg/day

F15 and F19 = 6 mg/kg/day

s.c. route most promising and consistent

[*]- Statistically different from negative control ($p < 0.001$)

[**]- Not statistically different between values ($p > 0.05$)

Clinical Trials: Experimentally infected dogs

Experimentally infected dogs (10^6 *L. infantum* (MCAN/PT/03/IMT335) amastigotes)

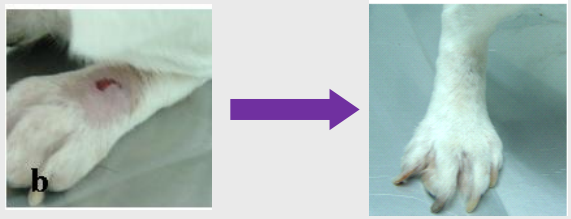
- Female beagle (3 years old, 12 Kg)

Treatment : 10 i.v. administrations of 10 mg TFL/kg/day (F15 = DOPC:DOPG)

- **Therapeutic Effect** (1 to 3 month after treatment)

Remission of clinical signs

(ulcerative lesions)



Reduction of parasite load

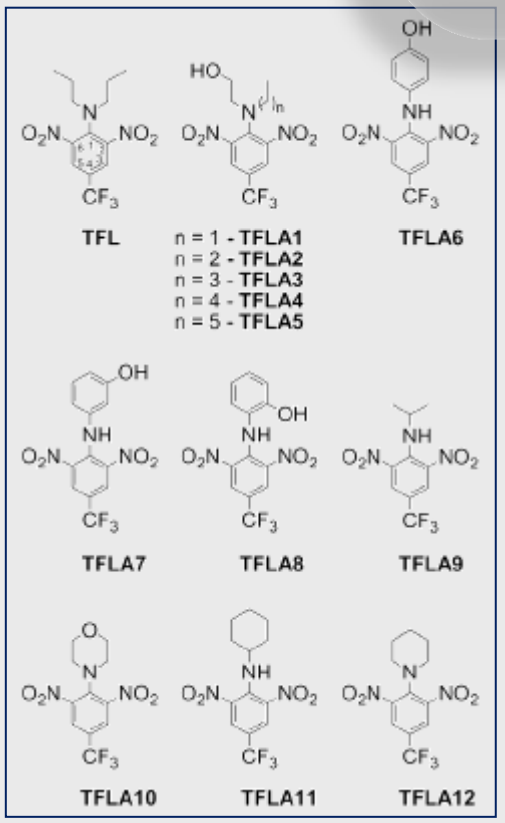
Time Line (month)		Parasites/g tissue	
		Bone Marrow	*Lymph Nodes*
5	after infection	1000	64000
1	after treatment	Negative	16000
3	after treatment	4000	16000

New Strategies: Dinitroanilines Derivatives (TFL-D)

Two Approaches = Chemical Modification + Incorporation in Liposomes

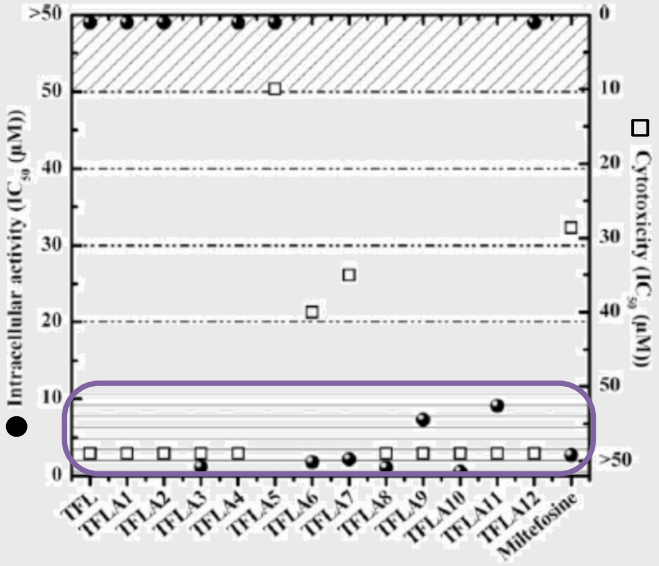
Compounds with improved physicochemical properties easier to administer *in vivo* (free or liposomal form)

TFL - Derivatives*



Biological Evaluation (*in vitro*)

- Cytotoxicity (IC_{50} > 50 µM)
- Haemolytic activity (HC_{50} < 10 µM)
- Intracellular activity (IC_{50} < 10 µM)



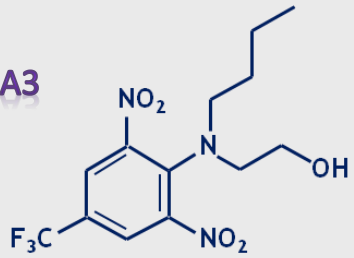
*Bioorganic & Medicinal Chemistry 18 (2010) 274–281

Synthesis and biological evaluation of trifluralin analogues as antileishmanial agents

M. A. Esteves [a], I. Fragiadaki [b], R. Lopes [c,d], E. Scoulica [b], M. E. M. Cruz [c,d,*]

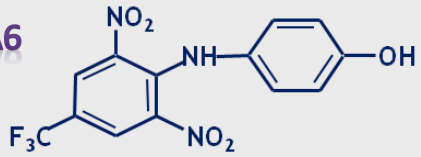
Liposomal Formulations of TFL-D

TFL-A3



2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-butylamino)-ethanol

TFL-A6



4-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol

Optimization of TFL-D liposomal formulations

- Lipid compositions with different membrane rigidity
- Effect of $[TFL\text{-}D/Lip]_i$ on incorporation parameters
- Stability studies of freeze-dried TFL-D formulations

Efficient incorporation of TFL-D

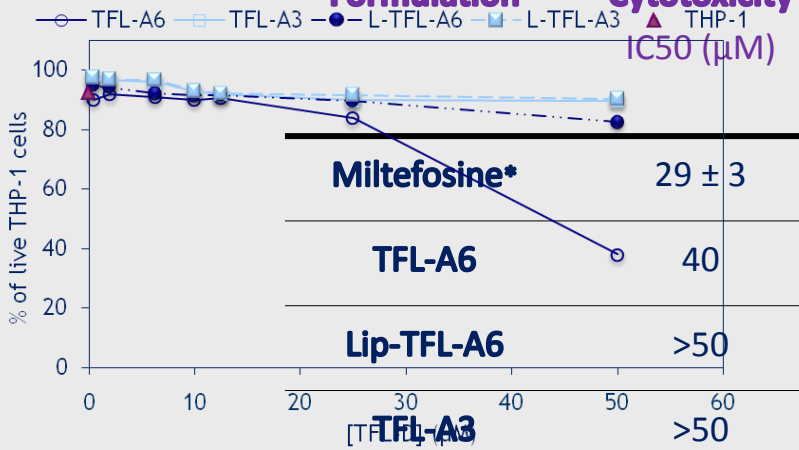
L.C. higher than TFL

TFL-D	DMPC:DMPG (molar ratio)	TFL-D: Lip (molar ratio)	L.C. (g/mol)	I.E. (%)	Mean Size (nm)	ζ (mV)
TFL-A6	7:3	1:4	77 ± 3	73 ± 3	186 ± 11	- 45 ± 5
TFL-A3	9:1	1:5	75 ± 9	91 ± 4	185 ± 14	- 31 ± 2

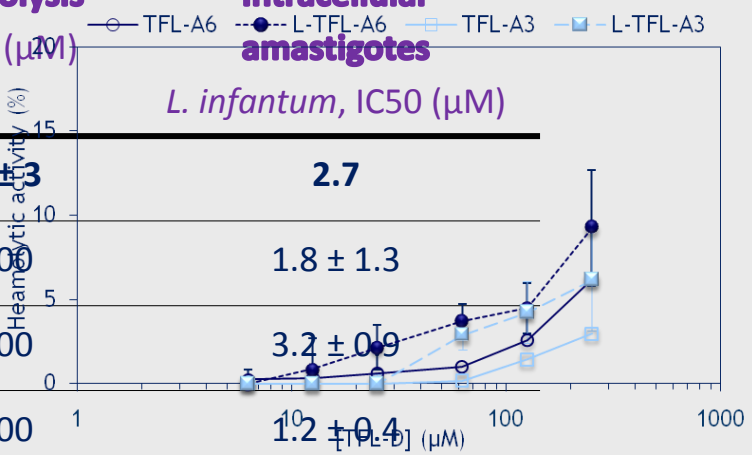
TFL-D biological evaluation *in vitro*

Free and liposomal

✸ **Cytotoxicity against THP-1 cells**



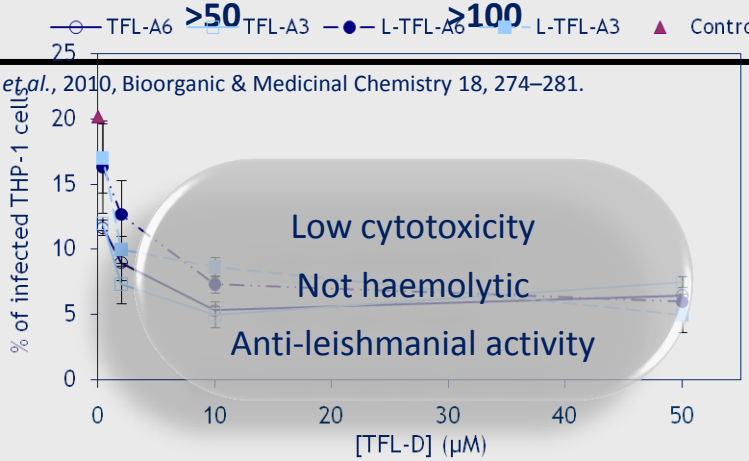
✸ **Haemolytic activity induced in RBCs**



Formulation	Cytotoxicity IC50 (µM)	Haemolysis HC50 (µM)	Intracellular amastigotes *L. infantum*, IC50 (µM)
Miltefosine*	29 ± 3	**38 ± 3**	**2.7**
TFL-A6	40	>500	1.8 ± 1.3
Lip-TFL-A6	>50	>500	3.2 ± 0.9
TFL-A3	>50	>500	1.2 ± 0.4
Lip-TFL-A3	>50	>500	2.4 ± 0.2
TFL*	>50	>100	>50

*Data from Esteves *et al.*, 2010, Bioorganic & Medicinal Chemistry 18, 274–281.

✸ **Anti-leishmanial activity against intracellular amastigotes of *L. infantum* in THP-1 infected cells**



Low cytotoxicity

Not haemolytic

Anti-leishmanial activity

Therapeutic Activity of TFL-D

Zoonotic Visceral Leishmaniasis

L. Infantum
MHOM/PT/89/IMT151

Infection
10^7 promast/mouse
(i.p.)

BALB/c

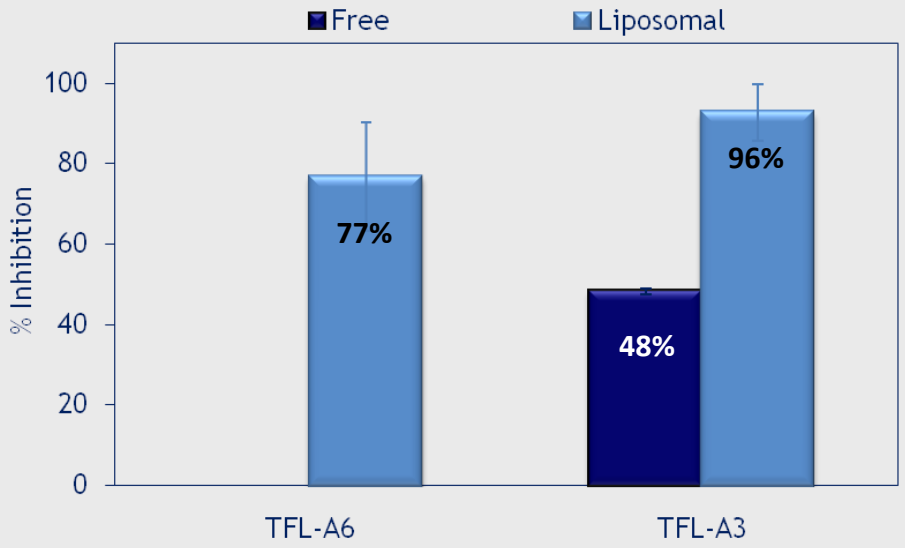
Treatment
45 to 56 days
post-infection

Spleen

LDA
15 days
post-treatment

Promastigote counting

Free *versus* Liposomal TFL-D anti-leishmanial activity



Liposomal TFL-D more active than the free compounds

Treatment = 10 i.p. administrations of 25 mg TFL-D/kg/day

Conclusions

- **Liposomal TFL is active *in vivo* against leishmaniasis**
  - **Visceral** leishmaniasis (70% parasite load inhibition);
  - **Cutaneous** leishmaniasis (58% reduction of lesions);
  - In experimentally **infected dogs** (improved their clinical condition; reduced the parasite load)
- **The Combined strategy of chemical modification and liposome incorporation produced new active Anti-leishmanial formulations**
  - ***In vitro***: Reduction of cytotoxicity; Maintenance of intracellular activity; Absence of haemolysis
  - ***In vivo*****:** Enhancement of anti-leishmanial activity
    - ↳ Higher than free TFL-D
    - ↳ Close to 100% parasite load inhibition
- **Liposomal formulations of Dinitroanilines**
  - Overcome difficulties of handling and administering *problematic* new drugs;
    - ↳ **Increase** water concentration;
    - ↳ **Systemic** administration without the need of toxic solvents
  - Provide efficient delivery to MPS cells in different tissues

Innovative approach for the treatment of *Leishmania* infections

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