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

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Neglected Tropical Diseases: Leishmaniasis

- 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.

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**Barriers**

- Penetration through cell membranes

**Issues**

- Low selectivity
- Repeated administrations
- High toxicity
- Poor patient compliance
- Emergence of resistance

**Drug Delivery Systems (Macrophages)**

**Drugs**

- Sub cellular localization of pathogen
- Sensitivity of the pathogen

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Antiparasitic and Antitumour drugs  8 and 9 of September 2011 | IBMC, Porto, Portugal | Host Institutions: I3S
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.
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

### Vesicular Concentric Bilayer Structures

- **Polar (hydrophilic) region**
- **Nonpolar (hydrophobic) region**

### LIPOSOMES PROPERTIES

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of bioactive agents</td>
<td>with different properties</td>
</tr>
<tr>
<td>Highly flexible systems</td>
<td></td>
</tr>
<tr>
<td>Penetrate and fuse with cells</td>
<td></td>
</tr>
<tr>
<td>Attachment of specific labels</td>
<td></td>
</tr>
<tr>
<td>Biodegradable/low toxicity</td>
<td></td>
</tr>
<tr>
<td>Large scale production</td>
<td></td>
</tr>
<tr>
<td>Long term stability (2 years)</td>
<td></td>
</tr>
</tbody>
</table>

### RATIONAL FOR INCORPORATION

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilization</td>
<td>Hydrophobic Bioactive Agent</td>
</tr>
<tr>
<td>Protection</td>
<td>Hydrophilic Bioactive Agent</td>
</tr>
<tr>
<td>Tailor made systems</td>
<td></td>
</tr>
<tr>
<td>Cellular internalization of the bioactive agent</td>
<td></td>
</tr>
<tr>
<td>Change the biodistribution</td>
<td></td>
</tr>
<tr>
<td>Pharmacologically acceptable</td>
<td></td>
</tr>
<tr>
<td>Other target ligands</td>
<td></td>
</tr>
</tbody>
</table>

**Vesicles**

Vesicular concentric bilayer structures are mainly made of phospholipids that present a **hydrophilic (polar) head** and **hydrophobic (non polar) fatty acid tail**. They are biocompatible, biodegradable, and non-immunogenic. They are tailored systems that can penetrate and fuse with cells, allowing cellular internalization of the bioactive agent. They are biodegradable, pharmaceutically acceptable, and have long-term stability of up to 2 years. Vesicles are drug carriers for a great variety of molecules, including small drug molecules, proteins, nucleotides, and plasmids.
## New Strategies: Dinitroanilines

**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

<table>
<thead>
<tr>
<th>Dinitroaniline</th>
<th>IC50 (µM) <em>L. infantum</em></th>
<th>Promastigotes</th>
<th>Amastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluralin</td>
<td>34,2</td>
<td>19,1</td>
<td></td>
</tr>
<tr>
<td>Oryzalin</td>
<td>11,1</td>
<td>11,8</td>
<td></td>
</tr>
<tr>
<td>Benfluralin</td>
<td>26,2</td>
<td>17,7</td>
<td></td>
</tr>
<tr>
<td>Pendimethalin</td>
<td>23,3</td>
<td>12,6</td>
<td></td>
</tr>
</tbody>
</table>

**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) is a dinitroaniline with demonstrated in vivo efficacy in murine models of cutaneous Leishmaniasis when applied topically as an ointment (Chan et al., 1993).

![Chemical structure of Trifluralin](image)

Formulate TFL for systemic application without the need of toxic solvents and at therapeutic doses.
**Therapeutic Activity of Liposomal TFL**

*Visceral Leishmaniasis*

- **L. donovani**
  - MHOM/ET/67/L82
- **Infection**
  - Female BALB/c
- **Treatment**
  - Liver
  - 5x i.v.; 15 mg TFL/kg/day
  - 1 s.c.; 15mg Sbv/kg

**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

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**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

**Female BALB/c**

Infection

2 X 10^7 prom/mouse

7 days

Nodule sizing

Treatment

6 doses

Nodule sizing

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**Effect of lipid composition and administration route**

![Graph showing the effect of lipid composition and administration route](image)

- **Neg Control**
- **Sb(V)**
- **F15**
- **F19**

**Administration route**

- **iv**
- **ip**
- **sc**

**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)
**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**

<table>
<thead>
<tr>
<th>Time Line (month)</th>
<th>Parasites/g tissue</th>
<th>Bone Marrow</th>
<th>Lymph Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 after infection</td>
<td></td>
<td>1000</td>
<td>64000</td>
</tr>
<tr>
<td>1 after treatment</td>
<td>Negative</td>
<td>16000</td>
<td></td>
</tr>
<tr>
<td>3 after treatment</td>
<td>4000</td>
<td>16000</td>
<td></td>
</tr>
</tbody>
</table>
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*

![Chemical structures of TFL and its derivatives](image)

* Biological Evaluation (in vitro)
- Cytotoxicity ($IC_{50} > 50 \mu M$)
- Haemolytic activity ($HC_{50} < 10 \mu M$)
- Intracellular activity ($IC_{50} < 10 \mu M$)

Synthesis and biological evaluation of trifluratin analogues as antileishmanial agents
M. A. Esteves, J. Fragiadakis, R. Lopes, E. Scoutica, M. E. M. Cruz*
Liposomal Formulations of TFL-D

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

- Optimization of TFL-D liposomal formulations
  - Lipid compositions with different membrane rigidity
  - Effect of [TFL-D/Lip], on incorporation parameters
  - Stability studies of freeze-dried TFL-D formulations

Efficient incorporation of TFL-D L.C. higher than TFL
## TFL-D Biological Evaluation *in vitro*

### Free and Liposomal

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cytotoxicity IC50 (μM)</th>
<th>Haemolytic Activity HC50 (μM)</th>
<th>Intracellular Activity L. infantum, IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miltefosine*</td>
<td>29 ± 3</td>
<td>38 ± 3</td>
<td>2.7</td>
</tr>
<tr>
<td>TFL-A6</td>
<td>40</td>
<td>&gt;500</td>
<td>1.8 ± 1.3</td>
</tr>
<tr>
<td>Lip-TFL-A6</td>
<td>&gt;50</td>
<td>&gt;500</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>TFL-A3</td>
<td>&gt;50</td>
<td>&gt;500</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>


- **Cytotoxicity against THP-1 cells**
- **Haemolytic activity induced in RBCs**
- **Intracellular amastigotes**
- **Anti-leishmanial activity against intracellular amastigotes of L. infantum in THP-1 infected cells**

### Graphs

1. Graph showing % of live THP-1 cells.
2. Graph showing Haemolytic activity (%).
3. Graph showing Anti-leishmanial activity.

**Legend:**
- ▲ THP-1
- TFL-A6
- L-TFL-A6
- L-TFL-A3
- TFL-A3
- L-TFL-A3

**Key Points:**
- Low cytotoxicity
- Not haemolytic
- Anti-leishmanial activity

**Antiparasitic and Antitumour drugs** 8 and 9 of September 2011 | IBMC, Porto, Portugal | Host Institutions: l3S
**Therapeutic Activity of TFL-D**

**Zoonotic Visceral Leishmaniasis**

- **L. Infantum**
  - MHOM/PT/89/IMT151

  - Infection: 10⁷ promast/mouse (i.p.)
  - BALB/c
  - Treatment: 45 to 56 days post-infection
  - Spleen
  - LDA: 15 days post-treatment
  - Promastigote counting

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

- **TFL-A6**: 77% inhibition
- **TFL-A3**: 96% inhibition

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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