Experimental models for lead optimisation of novel antileishmanial agents

Sunil Puri, PhD
Central Drug Research Institute
Lucknow, India
Experimental Models for Lead Identification of Novel Antileishmanial Agents

S K Puri

Central Drug Research Institute, Lucknow, India

WorldLeish4
Leishmaniasis - Lead Optimization Consortium

CDRI – DNDi - Advinus Therapeutics Pvt. Ltd.
Antileishmanial Experimental Models

- **Parasite:** *Leishmania donovani*

- **In vitro assays:**
  - Promastigote
  - Axenic Amastigotes
  - Intra-Macrophage Amastigote

- **In vivo model:**
  - Golden hamster
  - Mouse
**In Vitro Antileishmanial Assays**

- **Against Promastigote stage parasites**
  - Direct counting of promastigotes under microscope
  - MTT Assay and Alamar blue assay
  - Incorporation of Radiolabeled precursors
  - Flow cytometry using different fluorescent stains
  - Reporter genes viz. Green Fluorescent Protein or Luciferase transfected parasites

- **Against Amastigote stage parasites**
  - Axenic amastigotes
  - Amastigotes in mammalian macrophages-
    - Giemsa Staining
    - Use of reporter gene to monitor intracellular proliferation (e.g. β-galactosidase and firefly luciferase)
## Advantages and Limitations of Models

| **Promastigotes** | ♦ Not the relevant life cycle stage  
♦ Lack of correlation / predictability in data from promastigotes to amastigote stage |
| **Axenic amastigotes** | ♦ Assay does not test for penetration of compounds into the host cell nor for activity in macrophage phagolysosome  
♦ Different metabolic processes than in intracellular amastigotes |
| **Amastigotes in macrophages** | ♦ Labour intensive, subjective  
♦ HTS / MTS incompatibility |
| - **Giemsa Staining** | ♦ Rapid, sensitive, reproducible  
♦ Allows detection of only live, metabolically active cells by biphotonic imaging,  
♦ HTS / MTS compatible  
♦ HCS (ie. IPK) |
Requirements for an *In Vitro* Model

- Mammalian stage of the parasite
- A dividing population
- Quantifiable and reproducible measures of drug activity
- Activity of standard drugs in concentrations achievable in serum / tissue
- Adaptable to MTS / HTS
- Small amount of compound
- Low cost of test
Luciferase Assay

Principle
♦ Leishmania parasites transfected with gene encoded luciferase protein catalyses the mono oxygenation of beetle luciferin in presence of buffer containing Mg$^{2+}$, ATP and molecular oxygen.
♦ Results in production of oxyluciferin and light.
♦ Intensity of light is linearly related to the amount of luciferase and is measured using a Luminometer as RLU.

Luciferase Reaction

\[
\begin{align*}
\text{Beetle Luciferin} & \quad + \quad \text{ATP} + \quad \text{O}_2 \\
\text{Firefly Luciferase} & \quad \xrightarrow{\text{Mg}^{2+}} \\
\text{Oxyluciferin} & \quad + \quad \text{AMP} + \text{PP}_1 + \text{CO}_2 + \text{Light}
\end{align*}
\]
Advantages - Luciferase Assay

- Interpretation of results are easier.
- Determination of intracellular infection with and without drugs – Possible at a fraction of time.
- Permits to evaluate the toxicity of new compounds directly against the mammalian stage.
- All antileishmanial drugs tested are active against luciferase expressing amastigotes.
- Has the potential to be automated in 96 well formats for HTS / MTS
- Measures the total no. of parasites present while staining method provides an approximation of the macrophages that are counted.
In vitro evaluation: Amastigote-MQ Model by Luciferase assay

Day 1

J-774 A-1 cells

4x10^3 cells/100µl/well

96 well plate

Incubation: 24h, 37°C, 5% CO₂
Luc-Promastigote infection in J-774 cells

**Day 2**

- Luc-Promastigotes in stationary phase
- 6x10^4/ml
  - 100µl/well
- 1:15 (Cell : Promastigotes)
- Incubation: 24h, 37°C, 5% CO₂
- Infected cells (after 24 h)
- Blank
Preparation and dispensing of compounds

Stock in DMSO 10 mg/ml

Working soln. in media

Addition of compounds in 96 well plate 200µl/well

Untreated Control

Blank

Treated wells

Incubation at 37°C, 5% CO₂
Day 5

Change medium containing drugs

Day 6

Remove culture medium;
Add 50µl PBS
+ 50µl Steady Glo reagent per well

Inhibition > 70%
Identified for CC₅₀ / IC₅₀ Evaluation

Inhibition measured as RLU in treated wells

Inhibition < 70%
Not followed further

Record Luminiscence
**IC$_{50}$ value (µg/ml) of Reference drugs**  
**Luciferase assay versus Giemsa Staining**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Luciferase Assay</th>
<th>Giemsa staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Stibogluconate</td>
<td>57.30 ± 13.15</td>
<td>48.90 ± 10.20</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.015 ± 0.02</td>
<td>0.046 ± 0.02</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>6.19 ± 0.86</td>
<td>7.63 ± 2.45</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>13.68 ± 2.22</td>
<td>22.00 ± 6.60</td>
</tr>
</tbody>
</table>
Cytotoxicity Assay
Evaluation of CC$_{50}$ in J774 A-1 Cells

Day 1

J-774 A-1 cells
1x10$^5$ cells/100µl/well
96 well plate

Incubation: 24h, 37°C, 5% CO$_2$
Preparation and dispensing of compounds

DAY 2

Stock in DMSO
10 mg/ml

Working soln. in media

Untreated Control

Drug+DMSO

Highest Drug Conc.

Serial drug dilution in 96-well plate
200µl/well

Incubation: 72h, 37°C, 5% CO₂
Cytotoxicity Assay

After Incubation at 37°C for 2-3 hrs DMSO is added 150 µl/well Incubation for 15 min

Addition of MTT in PBS (5mg/ml) 25 µl / well

Absorbance recorded at 544 nm on micro plate reader

CC₅₀ values determined through preformed template (Werner & Koella, 1993)
Requirements for an In Vivo Model

- Animal models are expected to mimic the pathological features and immunological responses observed in human, when exposed to a variety of leishmania spp. with different pathogenic characters.

- More specifically, an immunologically appropriate model for VL would be in which cellular immunity is ineffective.

- Despite of many models developed, none accurately mimics what happens in humans.
**In Vivo Models for VL**

**Rodents**: Golden hamster  
Mouse strains

**Canines**: Dogs

**Simians**:

<table>
<thead>
<tr>
<th>New world monkeys</th>
<th>Old world monkeys</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aotus trivirgatus</em></td>
<td><em>Macaca mulatta</em> (Owl monkey)</td>
</tr>
<tr>
<td><em>Saimiri</em> sp.</td>
<td><em>Presbytis entellus</em> (Rhesus monkey)</td>
</tr>
<tr>
<td><em>Cercopithecus</em> sp.</td>
<td><em>Presbytis entellus</em> (Langur monkey)</td>
</tr>
<tr>
<td></td>
<td><em>Cercopithecus</em> sp. (Vervet monkey)</td>
</tr>
</tbody>
</table>
**In Vivo Model: Golden Hamster**

- *L. donovani* produces good infectivity in Golden hamsters after intra-cardiac inoculation with amastigote stage parasites.

- Host - parasite model is akin in histopathological features to VL in humans.

- Possible to perform multiple biopsies to monitor pre- & post treatment infection status in the same animal.

- Acceptable host for isolation and laboratory adaptation of clinical isolates.

- Animals develop Cachexia, a common symptom of terminal phase of disease, which is fatal to animals.

- Activity of known antileishmanial agents demonstrable in both liver as well as spleen.
**In Vivo Flow Chart: L. donovani in Golden Hamster**

- **Golden Hamsters**
  - Infection with $1 \times 10^7$ amastigotes/0.1ml

- **Parasite load assessment**
  - Splenic biopsy on day 14-16 p.i.

- **Evaluation at lower doses**

- **Treat - Day 2 post biopsy**
  - Dose: 50 mg/kg x 5 day;
  - Oral or i.p. route
  - 6 animals per dose

- **Biopsy Day 7 p.t.**
  - Monitor parasitic burden (Amastigote / 1000 nuclei)

- **Inhibition $\geq 80\%$**

- **Inhibition $< 80\%$**
  - Not followed further

- **Biopsy day 28 p.t.**
  - Monitor parasitic burden (Amastigote / 1000 nuclei)
In Vivo Efficacy Assessment

- Intensity of infection in both, treated and untreated animals, as also the initial count in treated animals is compared
- Efficacy expressed in terms of percentage inhibition (PI)

\[
PI = \frac{AT \times 100}{IT \times FI}
\]

where,

- \( PI \) = Percentage Inhibition of amastigotes’ multiplication.
- \( AT \) = Amastigotes number in post treatment biopsy.
- \( IT \) = Initial amastigote number in pretreatment biopsy.
- \( FI \) = Fold increase of parasites in untreated control animals

(Criteria for significant activity : \( PI > 80\% \))
### Efficacy of SSG: *L. donovani* in Hamsters

<table>
<thead>
<tr>
<th>Dose (mg/kg x 5) i.p.</th>
<th>Day 7 Post treatment (P.I.±SD) (n)</th>
<th>Day 28 Post treatment (P.I.±SD) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>98.8 ± 0.9 (6)</td>
<td>37.4 ± 8.9 (6)</td>
</tr>
<tr>
<td>40</td>
<td>89.4 ± 5.9 (6)</td>
<td>16.2 ± 10.1 (6)</td>
</tr>
<tr>
<td>20</td>
<td>68.8 ± 9.7 (6)</td>
<td>14.5 ± 4.4 (5)</td>
</tr>
<tr>
<td>10</td>
<td>54.6 ± 9.7 (6)</td>
<td>10.0 ± 5.0 (5)</td>
</tr>
<tr>
<td>5</td>
<td>31.8 ± 11.6 (6)</td>
<td>5.5 ± 5.0 (5)</td>
</tr>
</tbody>
</table>
## Efficacy of Miltefosine: *L. donovani* in Hamsters

<table>
<thead>
<tr>
<th>Dose (mg/kg x 5) oral</th>
<th>Day 7 Post treatment (P.I.±SD) (n)</th>
<th>Day 28 Post treatment (P.I.±SD) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>100 ± 0.0 (6)</td>
<td>93.2 ± 2.2 (6)</td>
</tr>
<tr>
<td>20</td>
<td>98.9 ± 0.27 (6)</td>
<td>83.12 ± 8.7 (6)</td>
</tr>
<tr>
<td>10</td>
<td>93.0 ± 4.9 (6)</td>
<td>43.1 ± 13.3 (5)</td>
</tr>
<tr>
<td>5</td>
<td>55.3 ± 5.5 (6)</td>
<td>26.4 ± 15.7 (5)</td>
</tr>
<tr>
<td>2.5</td>
<td>53.8 ± 13.4 (6)</td>
<td>3.3 ± 10.9 (5)</td>
</tr>
</tbody>
</table>
Team Members
Partnering along the path to deliver better treatments for visceral leishmaniasis

Challenges for and Potential in Early-Stage R&D
Wednesday, February 4, 2009 - Room A2: 11.00-13.00

DNDi
Drugs for Neglected Diseases initiative

LEAP
Leishmaniasis East Africa Platform