Experimental models for lead optimisation of novel antileishmanial agents

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Experimental Models for Lead Identification of Novel Antileishmanial Agents

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WorldLeish4
Leishmaniasis - Lead Optimization Consortium

CDRI – DNDi - Advinus Therapeutics Pvt. Ltd.

DNDi
Monitoring & Facilitator

Advinus
Synthesis & ADME

CDRI
Bio-evaluation
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
- Giemsa Staining
  - Labour intensive, subjective
  - HTS / MTS incompatibility
- Reporter gene assay
  - 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 catalyse the mono oxygenation of beetle luciferin in presence of buffer containing Mg2+, 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

```
Beetle Luciferin + ATP + O2 → Firefly Luciferase + Mg2+

Oxyluciferin

+ AMP + PP1 + CO2 + Light
```
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/100 µl/well

96 well plate

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

**Luc-Promastigotes in stationary phase**

**6x10^4/ml**
**100µl/well**

**Infected cells (after 24 h)**

**Incubation: 24h, 37°C, 5% CO₂**

**Day 2**

**Blank**

**1:15 (Cell : Promastigotes)**
Preparation and dispensing of compounds

**DAY 3**

- Stock in DMSO 10 mg/ml

- Working soln. in media

- Blank

- Untreated Control

- Treated wells

Addition of compounds in 96 well plate

- 200µl/well

- 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 measured as RLU in treated wells

Inhibition > 70%

Identified for CC₅₀ / IC₅₀ Evaluation

Inhibition < 70%

Not followed further

Record Luminiscence
# IC<sub>50</sub> 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

Serial drug dilution in 96-well plate

Untreated Control

Drug+DMSO

Highest Drug Conc.

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

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

DAY 5

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.

CC_{50} 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

<table>
<thead>
<tr>
<th>Rodents</th>
<th>Golden hamster Mouse strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canines</td>
<td>Dogs</td>
</tr>
<tr>
<td>Simians</td>
<td></td>
</tr>
</tbody>
</table>

**New world monkeys**
- *Aotus trivirgatus* (Owl monkey)
- *Saimiri sp.* (Squirrel monkey)

**Old world monkeys**
- *Macaca mulatta* (Rhesus monkey)
- *Presbytis entellus* (Langur monkey)
- *Cercopithecus sp.* (Vervet monkey)
**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

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

- **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 ≥ 80%**

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

- **Splenic biopsy on day 14-16 p.i.**
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)

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

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>
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</table>
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