


Experimental models for lead optimisation of novel antileishmanial agents

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DNDi
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Experimental Models for Lead Identification of Novel Antileishmanial Agents

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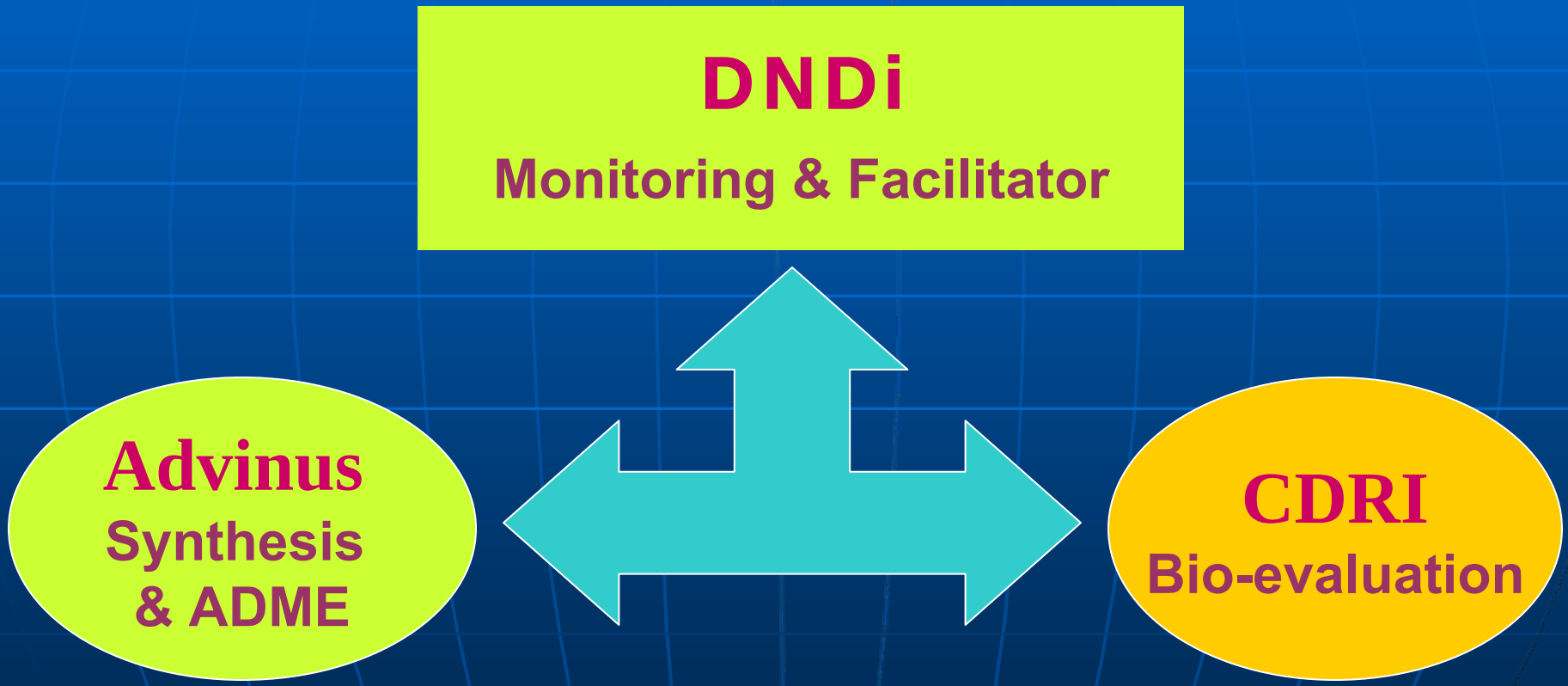
WorldLeish4

Leishmaniasis - Lead Optimization Consortium

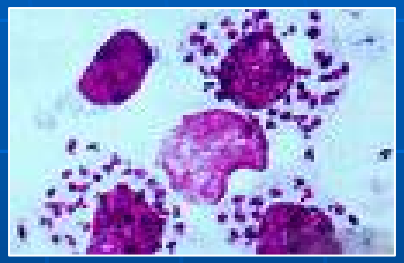
CDRI – DNDi - Advinus Therapeutics Pvt. Ltd.



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 Radiolabled 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. ß-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)

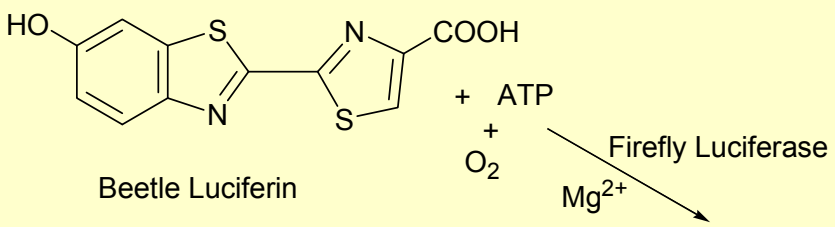
Requirements for an *In Vitro* Model

- 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

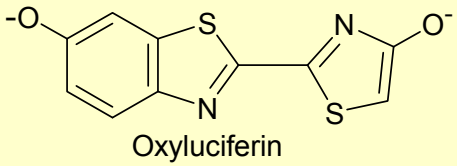
Luciferase Assay

Principle

- Leishmania parasites transfected with gene encoded luciferase protein catalyses 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



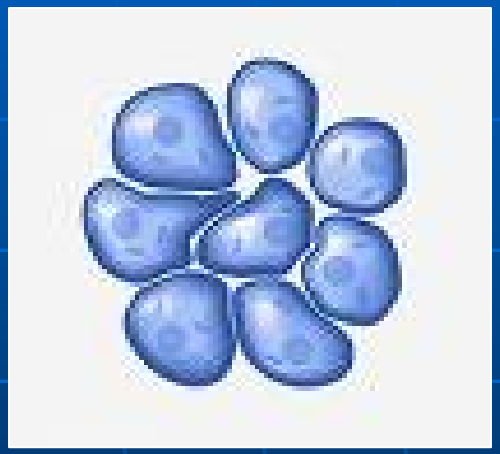
+ AMP + PP_1 + CO_2 + Light

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/100µl/well

96 well plate

Incubation: 24h, 37^0C, 5% CO_2

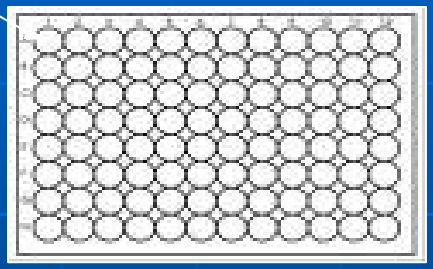
Luc-Promastigote infection in J-774 cells

Day 2

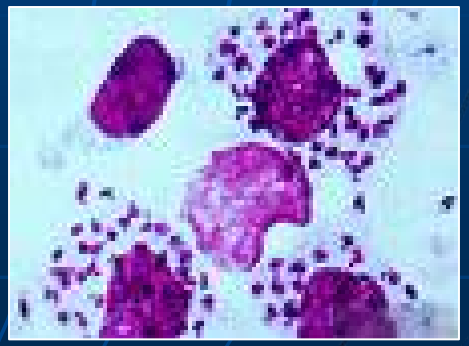
Luc-Promastigotes in stationary phase

$6x10^4$/ml
100µl/well

Blank



1:15
(Cell : Promastigotes)



Infected cells
(after 24 h)

Incubation: 24h, 37^0C, 5% CO_2

Preparation and dispensing of compounds

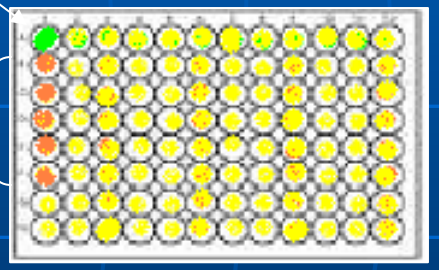
DAY 3

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 37^0C, 5% CO_2

Day 5

Change medium containing drugs

Day 6

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

Record Luminiscence

Inhibition measured as RLU in treated wells

Inhibition > 70%

Identified for CC_{50} / IC_{50} Evaluation

Inhibition < 70%

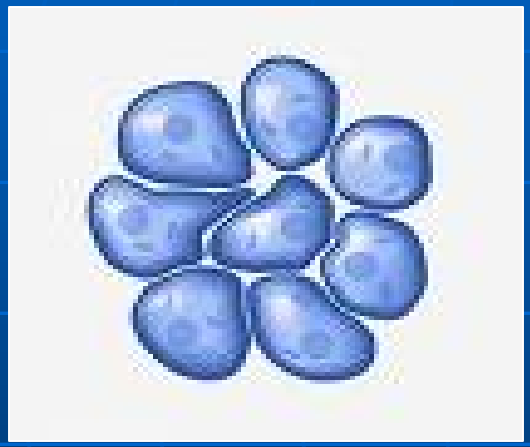
Not followed further

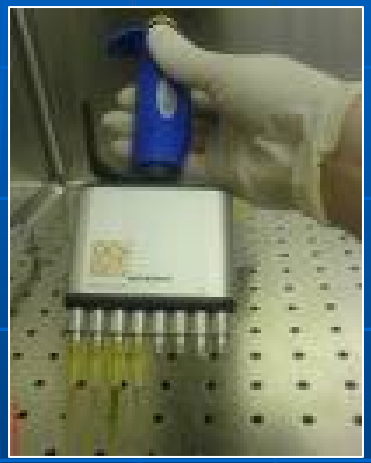
IC_{50} value (µg/ml) of Reference drugs Luciferase assay *versus* Giemsa Staining

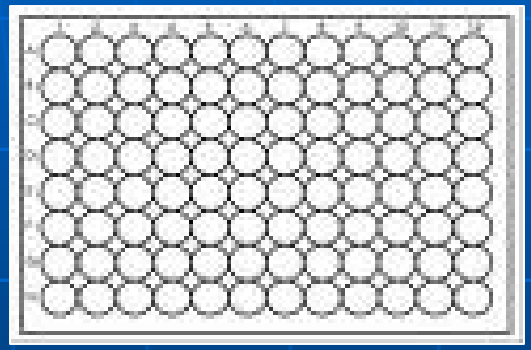
Drug	Luciferase Assay	Giemsa staining
Sodium Stibogluconate	57.30 ± 13.15	48.90 ± 10.20
Amphotericin B	0.015 ± 0.02	0.046 ± 0.02
Miltefosine	6.19 ± 0.86	7.63 ± 2.45
Pentamidine	13.68 ± 2.22	22.00 ± 6.60

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

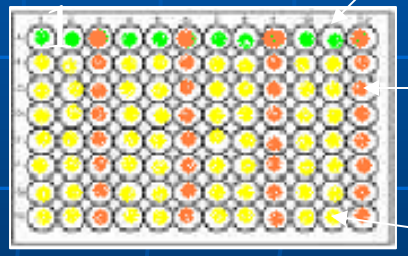
Incubation: 24h, 37^0C, 5% CO_2

Preparation and dispensing of compounds

DAY 2

Stock in DMSO
10 mg/ml

Working soln.
in media

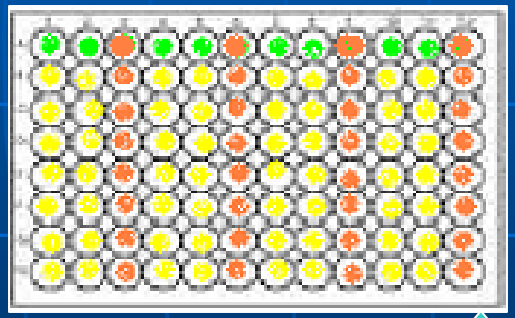


Serial drug dilution in 96-well plate
200µl/well
Incubation : 72h , 37^0C, 5% CO_2

Cytotoxicity Assay

DAY 5

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



Absorbance recorded at 544 nm on micro plate reader

After Incubation at 37^0C for 2-3 hrs DMSO is added 150 µl/well Incubation for 15 min

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

Rodents : **Golden hamster**
Mouse strains

Canines : **Dogs**

Simians :

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 $1x10^7$ amastigotes/0.1ml

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

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%

Not followed further

Biopsy day 28 p.t.
Monitor parasitic burden
(Amastigote / 1000 nuclei)

Inhibition ≥ 80%

Evaluation at lower doses

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

Dose (mg/kg x 5) i.p.	Day 7 Post treatment (P.I.±SD) (n)	Day 28 Post treatment (P.I.±SD) (n)
80	98.8 ± 0.9 (6)	37.4 ± 8.9 (6)
40	89.4 ± 5.9 (6)	16.2 ± 10.1 (6)
20	68.8 ± 9.7 (6)	14.5 ± 4.4 (5)
10	54.6 ± 9.7 (6)	10.0 ± 5.0 (5)
5	31.8 ± 11.6 (6)	5.5 ± 5.0 (5)

Efficacy of Miltefosine : *L. donovani* in Hamsters

Dose (mg/kg x 5) oral	Day 7 Post treatment (P.I.±SD) (n)	Day 28 Post treatment (P.I.±SD) (n)
40	100 ± 0.0 (6)	93.2 ± 2.2 (6)
20	98.9 ± 0.27 (6)	83.12 ± 8.7 (6)
10	93.0 ± 4.9 (6)	43.1 ± 13.3 (5)
5	55.3 ± 5.5 (6)	26.4 ± 15.7 (5)
2.5	53.8 ± 13.4 (6)	3.3 ± 10.9 (5)

Team Members


CENTRAL DRUG RESEARCH INSTITUTE

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

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