Malaria Parasite Biology and Drug Discovery Laboratory

In vitro antiplasmodial drug screening services

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Summary of services available to detect inhibitory effects of chemical matter on intraerythrocytic malaria parasites

1) A**sexual parasites** (pathogenic forms),
    a. Dual point primary screen (1&5 µM)
    b. Full dose-response (IC_{50})
    c. Cross-resistance against panel of genotyped drug sensitive/resistant parasite strains
    d. **SYBR Green I fluorescence readout or pLDH**

2) Gametocytes (sexual transmissible forms),
    a. Dual point primary screen (1&5 µM)
    b. Full dose-response (IC_{50})
    c. Luminescence (luciferase reporter lines) assay or pLDH

3) **Gametes (male and female gamete assay)**
    a. Dual point primary screen (1&5 µM)
    b. Full dose-response (IC_{50})

4) **Cytotoxicity**
    a. Activity against human HepG2 cell lines.

Part I:
Screening against asexual blood stage parasites

Methodology

General assay background

References:

1. Johnson, J. D., Dennull, R. A., Gerena, L., Lopez-Sanchez, M., Roncal, N. E., and Waters, N. C. (2007) Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening, *Antimicrob Agents Chemother* **51**, 1926-1933
2. Verlinden B, Niemand J, Snyman J, Sharma SK, Beattie RJ, Woster PM and L Birkholtz. (2011) Discovery of novel (bis)urea and (bis)thiourea-alkylated polyamine analogues with potent antimalarial activities. J Med Chem. Oct 13;54(19):6624-33.
3. Verlinden, B., Louw, A. I., Birkholtz, L. M., (2016) Resistaing resistance: is there a solution for malaria. Expert opin drug discov, Feb 26, 11(4):395-406.
4. Makler, M.T. , Hinrichs D.J., (1993) Measurement of lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitaemia. Am J. Trop Med Hyg. 48(2):205-210.

Screening of compounds against asexual *P. falciparum* parasites

Malaria parasite proliferation can be directly monitored in their intra-erytrocytic environment through detecting and monitoring either proliferation (DNA replication traced through SYBR Green I fluorescence). The following test cascade is used to identify and validate hit compounds.

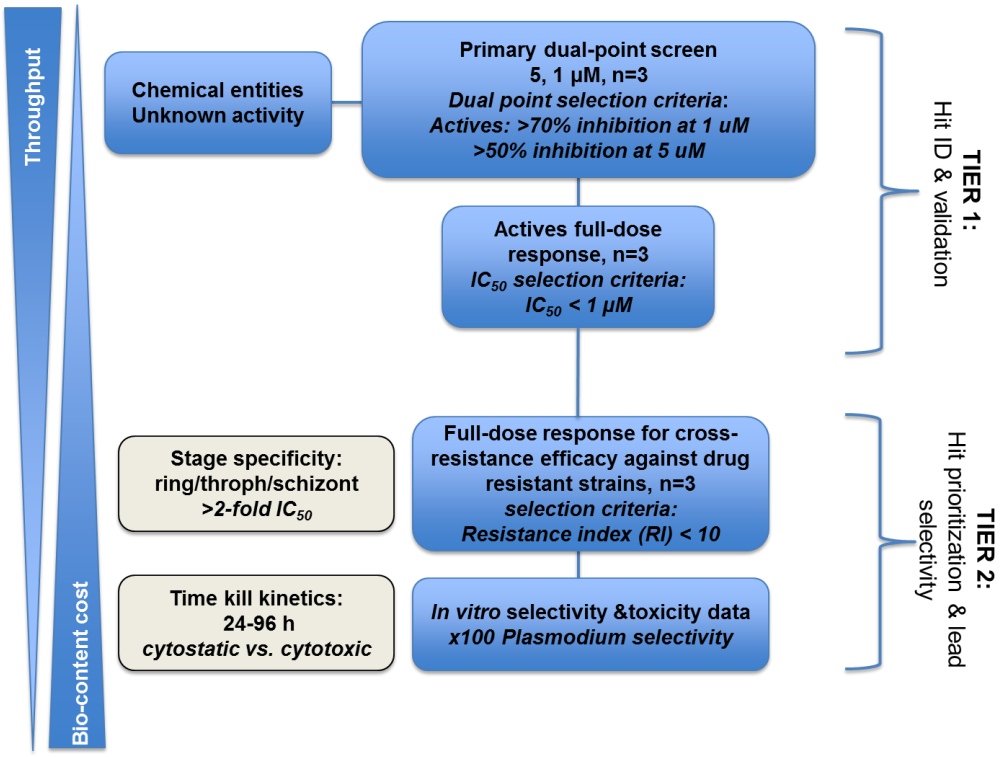

Figure 1: Asexual parasites screening cascade. The screening cascade indicates assay specifics (e.g. concentrations at which drugs are screened) as well as go/no-go selection criteria (italics). Essential screens are indicated in dark blue with parallel, investigative screens indicated in light blue.

Chemical matter requirements

- At least 1 mg of chemical matter is required for primary screens (and limited downstream assays)
- Information on MW, storage conditions and stabilities in various solutions is required.
- Compounds are routinely dissolved in DMSO and stored either at room temp or at 4°C, desiccated and in the dark, unless otherwise specified.
- Please see attached compound information sheet that needs to be completed with your request.

Primary screens

Test series or chemical matter is firstly screened at two concentrations (e.g. 1 and 5 uM or lower depending on chemical background) for one biological repeat, performed in triplicate. This is typically only performed against the standard, drug sensitive lab strains: 3D7 *P. falciparum* parasites or the parent population, NF54 *P. falciparum* parasites.

Hit selection criteria: >70% inhibition at 5 uM and >50% inhibition at 1 uM

Dose-response evaluation

Compounds passing primary screen hit criteria are further evaluated for full IC_{50} determination (concentration at which 50% inhibition of proliferation or viability is observed). This is typically only performed against the standard, drug sensitive lab strains: 3D7 *P. falciparum* parasites or the parent population, NF54 *P. falciparum* parasites. Data are represented from at least three independent biological replicates, each performed in technical triplicates.

Classification criteria

Compounds with antiplasmodial activities are classified as (www.mmv.org),:

Marginally active or inactive	IC_{50} > 10 µM
Moderately active	IC_{50} = 1 - 10 µM
Active	IC_{50} = 0.1 - 1 µM
Highly active	IC_{50} < 0.1 µM

Reference activities

Standard anti-malarials typically produce the following average IC_{50} values in our assays, and corresponds to literature (Duraisingh *et al.* 2000):

Chloroquine	15.7 ± 2.2 nM
Artemisinin	10.8 ± 4.0 nM

Cross-resistance evaluation

Various drug resistant *P. falciparum* parasites (ie. K1 full drug resistance, W2 chloroquine resistance or HB3 folate resistance, 7G8, D6 and Dd2) are included in a panel to provide information on possible resistance occurring (i.e. loss of activity) of hit compounds. Full dose-responses are determined against these strains for hit compound only. Resistance indices (RI) are used as a measure of efficacy.

Classification criteria:

RI < 10 = no cross-resistance
RI > 10 = potential resistance issues

Cost & Capacity

Dual point primary screens

96-well platform (DP)	Cost per plate (9 compounds can be tested/plate)	Cost per compound
	SYBR Green assay	SYBR Green assay
	Drug sensitive (3D7/NF54) strain	**Drug sensitive (3D7/NF54) strain**
Culture Cost	R 307.03	R 34.11
Assay Cost	R 1 157.32	R 128.59
Labour Cost	R 160.00 p/h x 2 per plate = R 320.00	R 17.80 p/h x 2 per plate = R 35.60
Total Cost, n=1	**R 1 784.35**	**R 198.30**
Total cost, n=3	**R 5 353.05**	**R 594.90**

Capacity for Dual points: 20 (cpds/2 months)
IC_{50} Screens: 20 (cpds/2 months)
Time for completion: 2 months
Hours of labour: 25 hours

Full IC_{50} determination

96-well platform (IC_{50})	Cost per plate (2 compounds can be tested/plate)	Cost per compound
	SYBR Green assay	SYBR Green assay
	Drug sensitive (3D7/NF54) strain	**Drug sensitive (3D7/NF54) strain**
Culture	R 307.03	R 153.93
Assay	R 1 157.32	R 578.70
Labour	R 320.00	R 160.00
Total Cost, n=1	**R 1 784.35**	**R 892.63**
Total Cost, n=3	**R 5 353.05**	**R 2 677.89**

Capacity for Dual points: 20 (cpds/2 months)
IC_{50} Screens: 20 (cpds/2 months)
Time for completion: 2 months
Hours of labour: 25 hours

Cross-resistance: Full IC_{50} determination

96-well platform (IC_{50})	Cost per plate (2 compounds can be tested/plate)	Cost per compound
	SYBR Green assay	SYBR Green assay
	Drug resistant (Dd2, HB3, K1, W2, D6 and 7G8) strains	**Drug resistant (Dd2, HB3, K1, W2, D6 and 7G8) strains**
Culture	R 307.03	R 153.93
Assay	R 1 157.32	R 578.70
Labour	R 320.00	R 160.00
Total Cost per strain, n=1	**R 1 784.35**	**R 892.63**
Total Cost per strain, n=3	**R 5 353.05**	**R 2 677.89**

Part II:
Screening against gametocytes

Background

The gametocyte assays performed at the M^{2}PL at UP, as part The South African Malaria Transmission-blocking Consortium (SAMTC), which has developed a comprehensive panel of robust validated *in vitro* screens that now permit small chemical entities to be assayed against key developmental stages representing *Plasmodium falciparum* transmission (Birkholtz *et al.*, Trends in Parasitology, 2016).

Cost & Capacity for gametocytocidal compound activity screens

Available gametocyte assays summary:

a. **Dual point screens (two concentrations, Luciferase)**
b. **Full IC_{50} determination (Luciferase)**

a. Dual Point primary screens

Gametocytocidal screens of compounds (% inhibition) are performed using at least two assay platforms depending on chemical background against *P. falciparum* strain NF54 at 1 and 5 µM or if more stringency is required, 1 and 0.5 µM. Available assay platforms include a reporter gene assay (luciferase reporter lines of NF54) (Reader *et al.,* Malaria Journal, 2015). Compounds are prioritised for IC_{50} determination based on the following selection criteria:

1) **Good activity** (IC_{50} expected to be below 1 µM)
   >70% inhibition at 5 µM and >50% inhibition at 1 µM
2) **Moderate activity** (IC_{50} expected to be between 1 and 5 µM)
   >70% inhibition at 5 µM and <50% inhibition at 1 µM
   <70% inhibition at 5 µM and >50% inhibition at 1 µM
   50 to 70% inhibition at 5 µM and <50% inhibition at 1 µM
3) **No/minimal activity** (IC_{50} expected to be above 5 µM)
   <50% inhibition at 5 µM and <50% inhibition at 1 µM

- Compounds with **good activity** will be prioritized for full IC_{50} determination (n=3).
- Compounds with **moderate activity** will undergo a single IC_{50} determination (n=1) as confirmation of dual-point results.

b. Full dose-response determination

Compounds that fulfil the dual point screen criteria are prioritised for full dose-response determination. Again, all three assay platforms can be used (Reader *et al.,* Malaria Journal, 2015). Compounds are classified by activity as follows:

- Compounds with **good activity** have IC_{50} values **below 1 µM** (n=3 biological replicates, each with technical triplicates).
- Compounds with **moderate active** have IC_{50} values **between 1 and 5 µM** (n=1 biological assay, with technical triplicates).

Cost & Capacity

Dual point primary screens: Early and Late stage gametocytes

96-well platform (DP)	Cost per plate (9 compounds can be tested/plate)	Cost per compound
	Luciferase assay	Luciferase assay
	Early stage gametocytes	**Early stage gametocytes**
Assay Cost	R 3 012.11	R 334.68
Labour Cost	R 320.00	R 35.60
Total Cost, n=1	**R 3 332.11**	**R 370.28**
Total cost, n=3	**R 9 996.33**	**R 1 110.84**

96-well platform (DP)	Cost per plate (9 compounds can be tested/plate)	Cost per compound
	Luciferase assay	Luciferase assay
	Late stage gametocytes	**Late stage gametocytes**
Assay Cost	R 3 255.01	R 361.66
Labour Cost	R 320.00	R 35.60
Total Cost, n=1	**R 3 575.01**	**R 397.26**
Total cost, n=3	**R 10 725.03**	**R 1 191.78**

IC_{50} determination: Early and Late stage gametocytes

96-well platform (IC_{50})	Cost per plate (2 compounds can be tested/plate)	Cost per compound
	Luciferase assay	Luciferase assay
	Early stage gametocytes	**Early stage gametocytes**
Assay Cost	R 2 881.10	R 1 440.45
Labour Cost	R 320.00	R 160.00
Total Cost, n=1	**R 3 201.10**	**R 1 600.45**
Total cost, n=3	**R 9 603.30**	**R 4 801.35**

96-well platform (IC_{50})	Cost per plate (2 compounds can be tested/plate)	Cost per compound
	Luciferase assay	Luciferase assay
	Late stage gametocytes	**Late stage gametocytes**
Assay Cost	R 3255.01	R 1 627.49
Labour Cost	R 320.00	R 160.00
Total Cost, n=1	**R 3 575.10**	**R 1 787.49**
Total cost, n=3	**R 10 725.03**	**R 5 362.47**

Capacity for Dual points: 10 (cpds/2 months)
IC_{50} Screens: 10 (cpds/2 months)
Time for completion: 2 months
Hours of labour: 25 hours

Part III
Cytotoxicity evaluation

Selectivity of compounds against $HepG_2$

Selectivity is be determined by screening compounds for activity against HepG2 cells (human hepatocellular liver carcinoma cells). To determine cytotoxicity, the BioVision LDH-Cytotoxicity Assay Kit II is used and selectivity indices evaluated (activity against parasite/activity against mammalian cell).

Reference activities (www.mmv.org):

Minimum requirement: selectivity indices of >10-100
Good selectivity: selectivity indices of >1000

Cost & Capacity

96-well platform (DP)	Cost per plate (9 compounds can be tested/plate)	Cost per compound
	LDH-Cytotoxicity Assay	LDH-Cytotoxicity Assay
	HepG2 cells	**HepG2 cells**
Assay Cost	R 4 993.57	R 554.84
Labour Cost	R 310.00	R 34.00
Total Cost, n=1	**R 5 303.57**	**R 588.84**
Total cost, n=3	**R 15 910.71**	**R 1 766.52**

96-well platform (IC_{50})	Cost per plate (2 compounds can be tested/plate)	Cost per compound
	LDH-Cytotoxicity Assay	LDH-Cytotoxicity Assay
	HepG2 cells	**HepG2 cells**
Assay Cost	R 4 993.57	R 2 496.78
Labour Cost	R 310.00	R 155.00
Total Cost, n=1	**R 5 303.57**	**R 2 651.78**
Total cost, n=3	**R 15 910.71**	**R 7 955.34**

Capacity for Dual points: 50 (cpds/3 months)
IC_{50} Screens: 20 (cpds/3 months)
Time for completion: 3 months
Hours of labour: 25 hours