

## Antimalarial drug discovery: efficacy models for compound screening (supplementary document)

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### 1. A Protocol for Antimalarial Efficacy Testing *in vitro*

This protocol for assessing compound efficacy against *Plasmodium falciparum in vitro* uses [<sup>3</sup>H]-hypoxanthine as a marker for inhibition of parasite growth<sup>1,2</sup>. Many alternative protocols exist, including ones based on microscopic detection of Giemsa-stained slides, assays based on production of parasite lactate dehydrogenase, and the use of flow cytometry<sup>3</sup>.

**Parasite strain:** Several well-characterized strains (see Supplemental Table 1) are available, either from academic laboratories or from [www.malaria.mr4.org](http://www.malaria.mr4.org) (reagents available to registered users). One recommendation would be to test activity against a drug-sensitive line such as 3D7 (West Africa), D6 (Sierra Leone) or D10 (Papua New Guinea), as well as a drug-resistant line such as W2 or Dd2 (both from Indochina), FCB (SE Asia), 7G8 (Brazil) or K1 (Thailand).

**Malaria Culture Media:** RPMI 1640 medium containing L-glutamine (Catalog number 31800, Invitrogen), 50 mg/liter hypoxanthine, 25mM HEPES, 10 µg/ml gentamicin, 0.225% NaHCO<sub>3</sub> and either 10% human serum or 0.5% Albumax I or II (purified lipid-rich bovine serum albumin, Invitrogen). Medium is typically adjusted to a pH of 7.3 to 7.4.

**Low Hypoxanthine Media:** Same as above except that the hypoxanthine concentration is reduced to 2.5 mg/liter.

Serum (as opposed to Albumax) is important for culturing fresh isolates, and for maintaining properties of cytoadherence and gametocyte production (the latter is required for transmission back to mosquitoes). Some strains also prefer to propagate in serum. Batch-to-batch variation is nonetheless a problem, with occasional batches not supporting robust parasite growth. Accordingly, many laboratory lines have been adapted to propagate in the presence of Albumax, which typically gives more consistent growth between batches (variation was a problem in the past, but now appears to have been resolved). Albumax appears to reduce both the rate at which erythrocytes deteriorate *in vitro*, and pH drift when cultures are exposed to ambient air (i.e. during tissue culture hood manipulations).

**Preparation of host erythrocytes:** Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 500  $\times g$  for 5 minutes in a swing-out rotor. Leukocyte-free erythrocytes are typically stored at 50% hematocrit (i.e. 1 volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately  $5 \times 10^9$  cells/ml).

**Parasite Culture Conditions:** *P. falciparum* asexual blood stage parasites are propagated at 37°C in malaria culture media at 3-5% hematocrit in a reduced oxygen environment (e.g. a custom mixture of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>). Lines can be conveniently cultured in 6-24 well tissue culture plates in a modular chamber (Billups-Rothenberg, Del Mar, CA, [www.brincubator.com](http://www.brincubator.com)), with plates containing sterile water on the bottom to increase humidity and minimize desiccation. These chambers can be suffused with the low O<sub>2</sub> gas and maintained at 37°C in an incubator designed to minimize temperature fluctuations. Parasites can also be cultured in flasks that are individually gassed, or alternatively placed in flasks that permit gas exchange through the cap (in which case the incubator needs to be continuously infused with a low O<sub>2</sub> gas mixture). Depending on the line, parasites typically propagate 3-8 fold every 48 hr, thus care must be taken to avoid parasite cultures attaining too high a parasitemia (i.e. percentage of erythrocytes that are parasitized) for healthy growth. Most lines grow optimally at 0.5 – 4% parasitemia. Parasites are most suitable for drug assays when they are 2-5% parasitemia, and mostly ring stages with few or no gametocytes.

**Compounds:** Compounds can often be dissolved in 100% dimethyl sulfoxide (DMSO) and stored at –80°C (or –20°C). Particle size of insoluble compounds can be reduced by ball-milling or sonication. For the drug assays, serial drug dilutions (either 2 $\times$  or customized) are made in low hypoxanthine medium (see above) and added to 96-well culture plates at 100  $\mu$ l per well. Drugs are added to columns 3-12 (test samples), with columns 1 and 2 reserved for wells with low hypoxanthine medium without compound. All drugs are typically tested in duplicate or triplicate for each parasite line. Once completed, plates are placed into their own modular chamber, gassed and placed at 37°C. These plates should be set up no more than a few hours prior to addition of the parasites.

**Drug Assay Conditions:** Parasites are diluted to a 2 $\times$  stock consisting of 0.6% to 0.9% parasitemia (depending on the growth rate of the line) and 3.2% hematocrit in low hypoxanthine medium, and 100  $\mu$ l are added per well already containing 100  $\mu$ l of low hypoxanthine medium with or without compound (present at different concentrations). Plates are then incubated in a gassed modular chamber at 37°C for 48 hr (some labs prefer 24 hr). After this time, 100  $\mu$ l of culture supernatant from each well is removed and replaced with 100  $\mu$ l of low hypoxanthine medium containing a final concentration of 7.5  $\mu$ Ci/ml of [<sup>3</sup>H]-hypoxanthine (1 mCi/ml stock, Amersham Biosciences). After a further 24 hr, the plates are placed at –80°C for at least 1 hr to freeze the cells. Plates are then thawed and the cells are harvested onto glass fiber filters (Wallac, Turku, Finland). Filters are dried for 30 minutes at 80°C, placed in sample bags (Wallac), and immersed in scintillation fluid (Ecoscint A; National Diagnostic, Atlanta, GA). Radioactive emissions are counted in a 1205 Betaplate reader (Wallac). Mean counts per minute (cpm) are generally in the range 20,000-60,000, with an acceptable minimum of 10,000. Percentage reduction in [<sup>3</sup>H]-hypoxanthine uptake is equal to  $100 \times ((\text{geometric mean cpm of no drug samples}) - (\text{mean cpm of test$

samples)) / (geometric mean cpm of no drug samples). Percentage reductions are used to plot percentage inhibition of growth as a function of drug concentrations. IC<sub>50</sub> values are determined by linear regression analyses on the linear segments of the curves (IC<sub>90</sub> values can also be determined by curve-fitting and can provide an useful measure of variation between experiments). Assays are typically repeated on two or three separate occasions. Within each experiment, standard deviations are typically less than 10% of the mean. Differences in parasite stages of development can lead to up to two-fold shifts in the IC<sub>50</sub> values between experiments; however, these differences rarely affect the overall relationships between the parasite lines in terms of their differences in drug response<sup>4-11</sup>.

**Supplemental Table 1. Standard *Plasmodium falciparum* strains.**

Name	Clone	Origin	Resistant to	Multiplication Rate	Reference
Dd2	Yes (from WR'82)	Indochina	CQ, QN, PYR, SDX	5-6	[3]
W2	Yes (from Indochina-3)	Indochina	CQ, QN, PYR, SDX	5-6	[4]
HB3	Yes	Honduras	PYR	4	[5]
3D7	Yes (from NF54)	Apparently West Africa	--	4	[5]
D6	Yes (from Sierra Leone-1)	Sierra Leone	--	4	[4]
D10	Yes	Papua New Guinea	--	4-5	[6]
CAMP	No	Malaysia	PYR	4-5	[7]
FCB	No	Apparently SE Asia	CQ, QN, CYC	7-9	[8]
7G8	Yes	Brazil	CQ, PYR, CYC	4-5	[9]
K1	No	Thailand	CQ, PYR	4-5	[10]

CQ, chloroquine; QN, quinine; PYR, pyrimethamine; SDX, sulfadoxine; CYC, cycloguanil.

Multiplication rate refers to increase in total numbers of viable parasites per 48-hr generation. These rates and the drug phenotypes refer to data from the Fidock laboratory (Albert Einstein College of Medicine, NY) and may not be the same elsewhere. See [www.ncbi.nlm.nih.gov/projects/Malaria/epid.html](http://www.ncbi.nlm.nih.gov/projects/Malaria/epid.html) for additional information on these and other *P. falciparum* strains.

## 2. Protocols for Antimalarial Efficacy Testing *in vivo*

These protocols are meant to facilitate early investigations into the *in vivo* efficacy of candidate antimalarial compounds, using the rodent parasite *P. berghei*. We nevertheless recognize that many different protocols exist, some involving different rodent malaria models (Supplemental Table 2).

### 1. Primary Biological Assessment of *in vivo* Antimalarial Efficacy using the *P. berghei* Rodent Malaria 4-Day Suppressive Test

**Parasite strain:** *Plasmodium berghei*, ANKA strain

**Standard Drugs:** Chloroquine (Sigma)  
Artemisinin (Sigma)

**Standard Conditions:**

**Mice:** NMRI mice, SPF, females,  $25 \pm 2$  g. Free from *Eperythrozoon coccoides* and *Haemobartonella muris*.

**Cages:** Standard Macrolon type II cages

**Maintenance:** Air conditioned with 22°C and 50-70% relative humidity, diet with *p*-aminobenzoic acid content of 45mg/kg, and water *ad libitum*

**Test procedure**

day 0 Heparinized blood is taken from a donor mouse with approximately 30% parasitemia, (i.e. 30% of the erythrocytes are parasitized), and diluted in physiological saline to  $10^8$  parasitized erythrocytes per ml. An aliquot of 0.2 ml ( $=2 \times 10^7$  parasitized erythrocytes) of this suspension is injected intravenously (i.v.) or intraperitoneally (i.p.) into experimental groups of 5 mice.

2-4 hr post-infection, the experimental groups are treated with a single dose of test compound (for example 30 or 50 mg/kg), normally by the i.p. or subcutaneous (s.c.) route. Other routes of application can also be tested, including i.v. and oral (p.o.). The compounds are prepared at an appropriate concentration, as a solution or suspension containing 7% Tween80/ 3% ethanol (see below). In a more advanced stage of screening, compounds can also be prepared in standard suspending vehicle (SSV, see below).

day 1 to 3 24 hr, 48 hr and 72 hr post-infection, the experimental groups of mice are treated again with the same dose and by the same route as on day 0.

day 4 24 hr after the last treatment (i.e. 96 hr post-infection), blood smears from all

Antimalarial efficacy screening: *in vitro* and *in vivo* protocols. Supplemental file.

animals are prepared and stained with Giemsa. Parasitemia is determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. For low parasitemias (<1%), up to 4000 erythrocytes have to be counted. Alternatively the parasitemia can also be determined by FACS analysis, which works particularly well for parasitemias >1%. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups is calculated and expressed as percent reduction (= activity) using the following equation:

$$\text{Activity} = 100 - \left\{ \frac{\text{mean parasitemia treated}}{\text{mean parasitemia control}} \times 100 \right\}$$

For slow acting drugs, additional smears should be taken on day 5 and day 6, the parasitemia determined and the activity calculated. Untreated control mice typically will die approximately one week after infection. For treated mice the survival-time (in days) is recorded and the mean survival time is calculated in comparison to untreated and standard drug treated groups. Mice still without parasitaemia on day 30 post-infection are considered cured. Observations concerning adverse effects due to the drug, including weight loss, are recorded.

#### 7% Tween80/ 3% ethanol:

Compounds are dissolved in 70% Tween 80 and 30% ethanol. This solution is further diluted 10-fold with distilled sterile water to result in a stock solution containing 7% Tween 80 and 3% ethanol.

#### Standard suspending vehicle (SSV):

Na-CMC (carboxy methylcellulose)	5.0 g
Benzyl alcohol	5.0 mL
Tween 80	4.0 mL
0.9% aqueous NaCl solution	1.0 L

SSV should be prepared one day before use. This solution is stable for 3 weeks at 4°C.

## **2. Secondary Biological Assessment.**

Compounds showing good *in vivo* activity in the primary 4-day suppressive test should be evaluated further in secondary *in vivo* models (Supplemental Box 1). Mice, maintenance, drug preparation and application, and assessment of parasitemia are the same as for the basic 4-day treatment protocol. These secondary *in vivo* methods include:

**Dose ranging test:** Here, compounds are tested at a minimum of 4 different doses (the “dose ranging, full 4-day test”, typically 100, 30, 10 and 3 mg/kg, by both s.c. and p.o. routes of administration). ED<sub>50</sub> and ED<sub>90</sub> values are calculated by plotting the log dose against probit activity (for example, using Microcal “Origin”). These reflect the drug concentrations at which 50% and 90% of suppression of parasitemia is achieved. Note that with short half-life drugs it may be necessary to dose multiple times per day. Two other parameters measured in this study are relative potency compared to an appropriate standard drug, and oral bioavailability (assessed by comparison of oral and parenteral administration). However,

bioavailability can be affected by formulation and may vary significantly within the same series of compounds, confounding interpretation of activity. For example, a recent study on artemisinin derivatives showed > ten fold variation in efficacy depending on the formulation <sup>12</sup>.

**Onset of activity and recrudescence test:** For this, a single 100 mg/kg dose is administered on day 3 post-infection by the s.c. route. Control mice receive the suspension vehicle alone as a placebo control. Beginning after 12 hours, 24 hours and extending to day 33, tail blood smears are taken daily and Giemsa stained, followed by an assessment of parasitemia. Results are expressed as the rapidity of onset of activity (disappearance of parasitemia), time point of recrudescence, increase of parasitemia and survival in number of days.

**Prophylactic test:** Once a therapeutic lead has been established, it is important to establish prophylactic activity. For this, mice are administered 100 mg/kg doses at -72 hr, -48 hr, -24 hr and 0 hr relative to the time of infection. Smears are examined daily to assess suppression of parasitemia, and survival times are measured in days.

For all these assays, CQ can be used as the reference drug. Typically, CQ has an ED<sub>50</sub> value of 1.5 – 1.8 mg/kg for *P. berghei* ANKA when administered s.c. or p.o. respectively, as determined in the full 4-day suppressive test (data not shown).

### 3. Tertiary Biological Assessment.

**Cross-Resistance:** For many antimalarial drug discovery projects, which depend on refining existing antimalarials or synthesizing new antimalarials that act upon the same target as an existing drug, the question of cross-resistance is paramount. Many drug-resistant strains of rodent malaria parasites have been selected over the past four decades by Professor Wallace Peters (Northwick Park Institute for Medical Research, Middlesex, UK). This includes 27, 7 and 14 lines of different drug resistance profiles for *P. berghei*, *P. yoelii* and *P. chabaudi* respectively. Some of these strains are available from MR4 ([www.malaria.mr4.org](http://www.malaria.mr4.org)). Cross-resistance studies employ the 4-day suppressive test described above, in order to determine whether the same ED<sub>50</sub> and ED<sub>90</sub> values are observed in drug-resistant strains of rodent malaria when compared to *P. berghei* ANKA.

***In vivo* generation of drug resistance:** It may also be helpful to determine the potential of parasites to develop resistance to a new compound *in vivo*, although the clinical relevance of these assays is unknown. Several high dose and incremental increase approaches have been used to select resistant lines. Of these, a widely used 2% relapse method <sup>13</sup> has proven to be useful for most classes of compounds. This entails administering a dose of compound that when given 1 hr before infection, delays the development of 2% parasitaemia until about 7-10 days post-infection. When the 2% level is reached, parasites are again passaged into fresh groups of mice 1 hr after administering the same dose of compound. The time to reach 2% parasitaemia is monitored on a daily basis. The degree of resistance is expressed as the reduction in “delay time to 2%”. In rodent models, the rate of selection of resistance varies from 90% within 10 days for atovaquone to 50% after 300 days for artemisinin <sup>13</sup>. The method can then be extended beyond this indicator of selection of resistance to demonstrate the stability of resistance following removal of drug pressure and to select a partner compound(s) that can slow the rate of development of resistance.

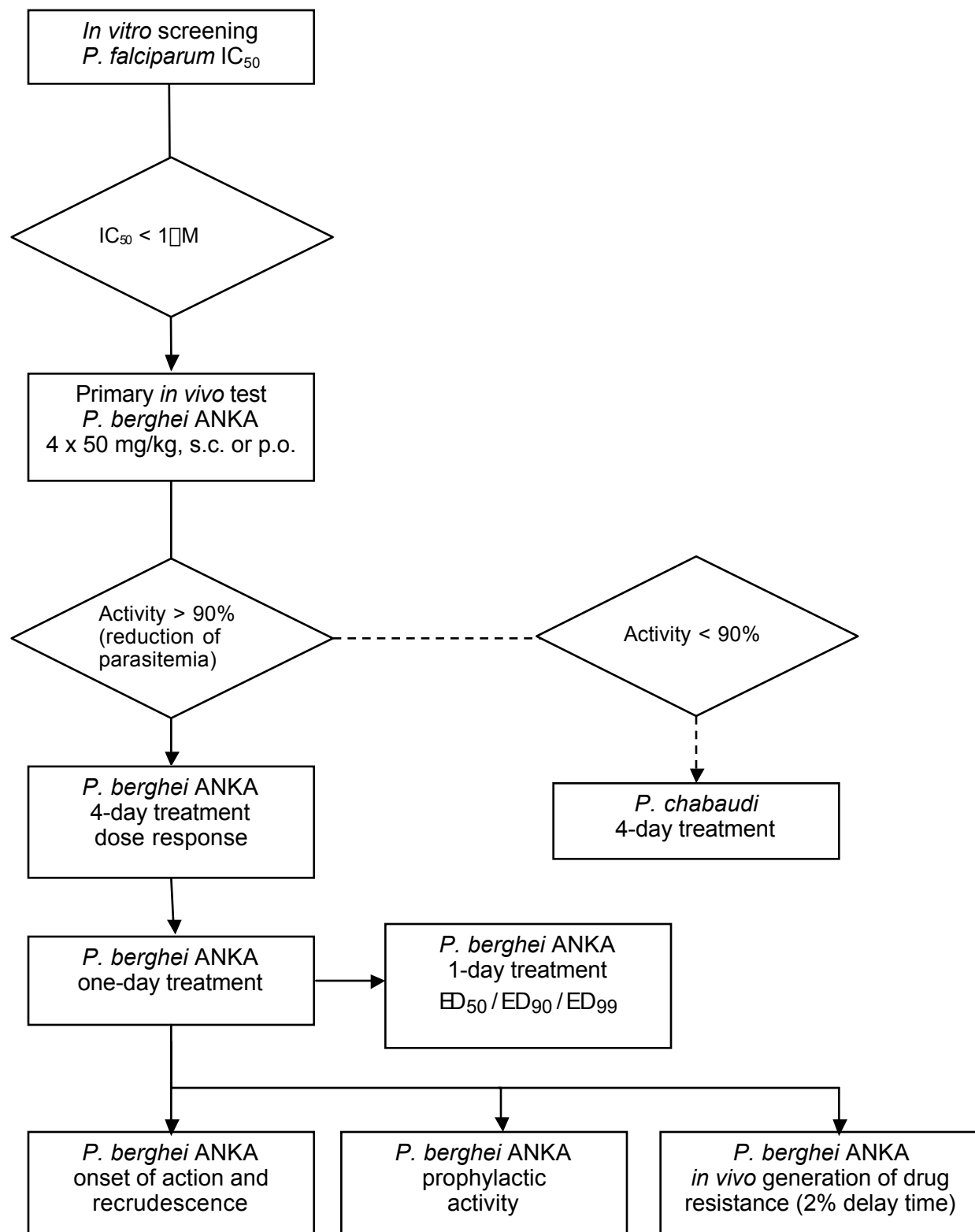
**Supplemental Table 2. Characteristics of rodent malaria infections.**

	<i>Plasmodium</i> species			
	<i>P. berghei</i>	<i>P. yoelii</i>	<i>P. chabaudi</i>	<i>P. vinckei</i>
First isolated	1948 (Zaire)	1965 (CAR)	1965 (CAR)	1952 (Zaire)
Cycle	asynchronous	asynchronous	synchronous	synchronous
Periodicity	22-25 hours	22-25 hours	24 hours	24 hours
Host cells	reticulocytes	reticulocytes	Mature RBC	Mature RBC
Mz per schizont	12 to 18	12 to 18	6 to 8	6 to 12
Primary use	Drug screening	Liver stage biology and vaccine studies	Mechanisms of drug resistance & antigenic variation	

Mz, merozoites; CAR, Central African Republic; RBC, red blood cells.

See [www.ncbi.nlm.nih.gov/projects/Malaria/Epid/Originlist/rodstrain.html](http://www.ncbi.nlm.nih.gov/projects/Malaria/Epid/Originlist/rodstrain.html) (compiled by Dr. David Walliker) for further information.

**Supplemental Box 1. Flow chart of one scenario for *in vivo* screening for antimalarial activity.**





## REFERENCES:

1. Desjardins, R. E., Canfield, C. J., Haynes, J. D. & Chulay, J. D. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* **16**, 710-8 (1979).
2. Fidock, D. A., Nomura, T. & Wellems, T. E. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol. Pharmacol.* **54**, 1140-7 (1998).
3. Noedl, H., Wongsrichanalai, C. & Wernsdorfer, W. H. Malaria drug-sensitivity testing: new assays, new perspectives. *Trends Parasitol* **19**, 175-81 (2003).
4. Wellems, T. E. et al. Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. *Nature* **345**, 253-255 (1990).
5. Oduola, A. M., Weatherly, N. F., Bowdre, J. H. & Desjardins, R. E. *Plasmodium falciparum*: cloning by single-erythrocyte micromanipulation and heterogeneity *in vitro*. *Exp Parasitol* **66**, 86-95 (1988).
6. Walliker, D. et al. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* **236**, 1661-1666 (1987).
7. Culvenor, J. G. et al. *Plasmodium falciparum*: identification and localization of a knob protein antigen expressed by a cDNA clone. *Exp Parasitol* **63**, 58-67 (1987).
8. Schmidt, L. H., Harrison, J., Rossan, R. N., Vaughan, D. & Crosby, R. Quantitative aspects of pyrimethamine-sulfonamide synergism. *Am J Trop Med Hyg* **26**, 837-49 (1977).
9. Joy, D. A. et al. Early origin and recent expansion of *Plasmodium falciparum*. *Science* **300**, 318-21 (2003).
10. Burkot, T. R., Williams, J. L. & Schneider, I. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. R. Soc. Trop. Med. Hyg.* **78**, 339-341 (1984).
11. Thaithong, S. & Beale, G. H. Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. *Trans. R. Soc. Trop. Med. Hyg.* **75**, 271-273 (1981).
12. Peters, W., Fleck, S. L., Robinson, B. L., Stewart, L. B. & Jefford, C. W. The chemotherapy of rodent malaria. LX. The importance of formulation in evaluating the blood schizontocidal activity of some endoperoxide antimalarials. *Ann Trop Med Parasitol* **96**, 559-73 (2002).
13. Peters, W. & Robinson, B. L. The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. *Ann Trop Med Parasitol* **93**, 325-9 (1999).