

ten individuals, stained in 1% mercurochrome in water. Mosquitoes were given a second (uninfected) blood meal to promote sporozoite invasion of the salivary glands. Salivary gland sporozoites were isolated from 12 days post feeding onwards.

Isolation and maintenance of primary rhesus hepatocytes. Rhesus monkey hepatocytes were isolated from liver lobes as follows. Immediately after resection, the liver was flushed with Viaspan (Bristol-Meyers-Squibb) to maintain hepatocyte viability. Livers were processed within two hours from resection by the two-step collagenase protocol, as described by Guguen-Guillouzo *et al.* (3). After collagenase VIII (C2139, Sigma) digestion of hepatocytes, dead and non-hepatocyte cells were removed using 36% percoll gradient in DMEM. Cells were washed in William's E containing 10% fetal calf serum (FCS) before resuspending in William's B medium (William's E with glutamax, containing 10% FCS, 2% penicillin/streptomycin (P/S), 1% insulin/transferrin/selenium, 1% NaPyruvate, 1% MEM-NEAA, 50 μ M β -mercapto-ethanol, 10⁻⁷M dexamethasone (Alfasan, Woerden, The Netherlands)).

Cells were seeded at 290,000 cells/cm² into collagen coated (5 μ g/cm² rat tail collagen I) 96-well Greiner cellstar plates (90,000 cells/well) or 8-well Labtek chamber slides (210,000 cells/well) in William's B medium and left to attach for at least 2 h in a 37°C incubator at 5% CO₂. When attached, the medium was replaced by William's B with 2% dimethylsulfoxide (DMSO) to prevent hepatocyte dedifferentiation. Sporozoite infections were performed within three days after hepatocyte isolation.

Sporozoite inoculation of primary rhesus hepatocytes. Sporozoite inoculation of primary rhesus hepatocytes was performed according to Mazier *et al.* (4) between 14 and 28 days after feeding the mosquitoes on *P. cynomolgi* infected blood. Mosquitoes were transferred into beakers covered with mosquito nets, soaked in 70% ethanol and washed 4 times in Leibovitz' L15 medium containing 3% FCS and 2% P/S. Salivary glands were dissected under a Leica EZ4 stereo microscope, collected in the above medium in sterile 2 mL glass potter tubes on ice and ruptured by pottering 10 times. The suspension was passed over a 40 μ m filter and spun for 3 min at 13,000 rpm in an Eppendorf centrifuge to collect sporozoites, which were washed and resuspended in William's B medium and counted in a Bürker-Turk counter chamber after settling in a humidified box for at least 5 min. Hepatocytes were washed with William's B prior to sporozoite inoculation, which were added at 50,000 sporozoites/well for 96-well plates or at 90,000 sporozoites/well for LabTek chamber slides. 96-well plates were spun 10 min at 500xg and placed into a 37°C, 5% CO₂ incubator for at least three hours before the first medium refreshment. LabTek chamber slides were left at room temperature for 2 h to settle the sporozoites, and placed into a 37°C, 5% CO₂ incubator overnight. Medium was refreshed every other day. To evaluate the development of *P. cynomolgi* liver stages, slides were fixed by cold methanol fixation at indicated time points.

Drug assays on liver stage parasites. Compound stocks (10 or 20 mM stocks in DMSO) were aliquoted and stored at -20°C. Compounds were diluted in William's B medium, passed over a 0.2 μ m filter and further diluted to 10, 1 and 0.1 μ M. Atovaquone, primaquine and medium only were used as controls. For IC₅₀ determination two-fold serial dilutions (10-0.04 μ M final

concentration) were evaluated in duplicate. Test compounds were added to the EEF cultures at the time of first medium exchange. All medium refreshments contained the appropriate compound dilutions. Liver stage parasite numbers were determined as described below.

Visualisation of liver stages. Intracellular *P. cynomolgi* malaria parasites were stained with the cross-reactive mouse polyclonal anti-*P. falciparum* Hsp70.1 antibodies 1:2,000 in PBS (25). FITC labeled Goat-anti-mouse IgG was used as secondary antibody, 1:200 in PBS containing 2 μ M 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. Alternatively, anti-*P. cynomolgi* Hsp70.1 (anti-*Pcy*Hsp70) antibodies were used. These were generated by overexpression of part of the Hsp70 gene (amino acid 350-686, see supplementary data) in analogy to previous papers (5). Briefly, the *P. cynomolgi* Hsp70.1 gene was identified from the preliminary *P. cynomolgi* genome, by homology search with *P. yoelii* Hsp70. An *E. coli* codon optimized synthetic gene containing a N-terminal His-tag and Thrombin sites was overexpressed in *E. coli* by IPTG induction and purified via standard procedures. The protein was used for immunization of 2 rabbits and 2 mice according to "High speed immunization protocol" (Biogenes, Germany). Rabbit anti-*Pcy*Hsp70 antibodies were used in 1:10,000 dilution, FITC-labeled goat-anti-rabbit IG was used as secondary antibody, 1:200 in PBS with 2 μ M DAPI. The number of EEF was determined for each well using a Leica DMI6000 inverted microscope at 400x magnification, counting approximately 20,000 cells. In later assays, the number of intracellular parasites was determined using a high-content imaging system (Operetta®, Perkin-Elmer), the analysis protocol is available as supplementary data (Suppl.protocol1). Validation of the Operetta was performed by comparing manual counting results of a number of plates with the outcome of Operetta analysis. The PVM was visualized using rabbit anti-*Pcy*ETRAMP serum. *Pcy*ETRAMP was identified from the preliminary *P. cynomolgi* genome based on orthology with PF10-0164 (ETRAMP10.3). Two rabbits were immunized with a mixture of two peptides ((C)-IISPNDKKEGLD and (C)-IMKHKKERKEMED), according to "High speed peptide immunization protocol" (Biogenes, Germany). For Etramp-staining of the PVM, PFA-fixed hepatocytes were partially permeabilized with 0.5% digitonin. Rabbit anti *Pcy*ETRAMP was used in 1:500 dilution, together with mouse anti-*Pcy*Hsp70 (1:10,000). As secondary antibodies TRITC-labeled anti Rabbit IgG and FITC-labeled anti-mouse IgG were used, in 1:200 dilution in PBS containing 2 μ M DAPI.

Criteria for distinguishing large and small EEF parasites. Using the limited information available about *in vivo* hypnozoite morphology, we used the following definitions ((6), (7), (8) and (9)): "Hypnozoite": intracellular parasite with 1 nucleus and small round shape, maximally 7 μ m in diameter; "Developing parasite": intracellular parasite with more than 1 nucleus, larger than hypnozoite and round or irregular shape.

Statistical analysis. Correlations between variables were calculated using the non-parametric Spearman's Rho. IC₅₀ values for compounds were estimated with general least squares regression using the following equation: Percentage versus untreated = $100 * 1 / [1 + \text{Exp}((\text{Ln IC}_{50} - \text{Ln Compound}) * \text{Slope})]$. Where IC₅₀ = the concentration of compound required for 50% parasite death, Compound = the concentration of the compound, and Slope = a parameter indicating the steepness of the curve, with higher (absolute)

values indicating steeper curves. P-values smaller than 0.05 were considered significant.

Operetta data acquisition and analysis

Counting *P. cynomolgi* liver stage parasites using Operetta (Perkin-Elmer), image acquisition and analysis

Image acquisition:

DAPI (excitation 360-400, dichroic 405, emission 410-480)

Fluorescein (excitation 460-490, dichroic 495, emission 500-550)

Autofluo (excitation 460-490, dichroic 495, emission 560-630)

Analysis Operetta:

Input: Image (individual plane)

Find nuclei (DAPI), method C

Find Image region

Method: common Threshold

Channel: Fluorescein

Threshold: 0.85

Output population: Image Region FITC

Calculate intensity properties (1)

Channel: Fluorescein

Population: Image Region FITC

Region: Image Region

Output properties: Intensity Image Region FITC

Calculate intensity properties (2)

Channel: Autofluo

Population: Image Region FITC

Region: Image Region

Output properties: Intensity Image Region Autofluo

Calculate properties

Population: Image region

Method: by Formula: a/b

a= Intensity Image Region FITC

b= Intensity Image Region Autofluo

Output properties: Ratio FITC/AF

Calculate intensity properties (3)

Channel: Dapi

Population: Image Region FITC

Region: Image Region

Output properties: Intensity Image Region Dapi

Select Population (1)

Population: Image Region FITC

Method: filter by property

Filter F1: Ratio FITC/AF>5

Filter F2: Intensity Image Region Dapi>50

Filter F3: Intensity Image Region FITC>800

Output population: Ratio FITC/AF>5, Dapi>50, FITC>800

Calculate morphology properties:
Population: Ratio FITC/AF>5, Dapi>50, FITC>800
Region: Image Region
Method: standard

Output properties: parasite size

Select population (2)
Population: Ratio FITC/AF>5, Dapi>50, FITC>800
Method: Filter by property
Filter F1: parasite size>12

Output population: total parasites

Select population (3)
Population: Ratio FITC/AF>5, Dapi>50, FITC>800
Method: Filter by property
Filter F1: parasite size>12
Filter F2: parasite size<30

Output population: small EEF

Select population (4)
Population: Ratio FITC/AF>5, Dapi>50, FITC>800
Method: Filter by property
Filter F1: parasite size>=30

Output population: large EEF

Define results:

#nuclei

objects FITC/AF>5, Dapi>50, FITC>800

objects: total parasites

objects: small EEF
parasite size (μm^2) [mean size of small EEF]

objects: large EEF
parasite size (μm^2) [mean size of large EEF]

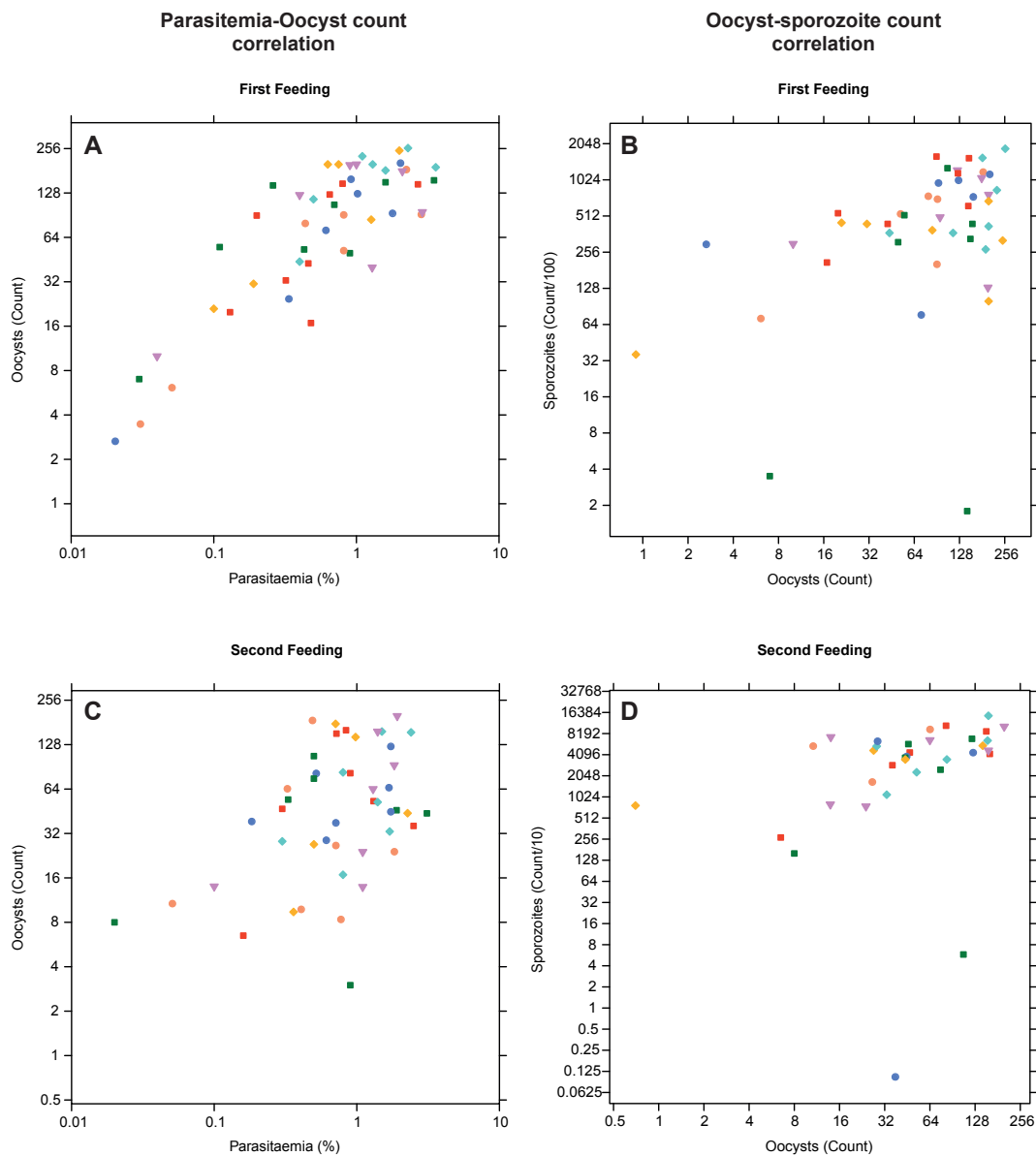


Figure S1: Correlation plots of parasitemia vs oocyst number and oocyst number vs sporozoite yield.

Panels A and C: Parasitemia calculations (determined from Giemsa stained thin film smears), vs oocyst numbers (mean of 10 mosquitoes) for feed 1 and 2. Panels B and D: oocyst numbers vs sporozoite numbers for feed 1 and 2. Oocyst count was positively correlated with blood stage parasitemia at the time of mosquito feeding (Spearman's Rho) 0.732, $p < 1.10^{-8}$ for feed 1 and 0.372 $p=0.009$ for feed 2. The number of sporozoites was weakly positively correlated with the amount of oocysts (Spearman's Rho) 0.398, $p = 0.0062$, for feed 1 and 0.514 $p=0.0017$ for feed 2

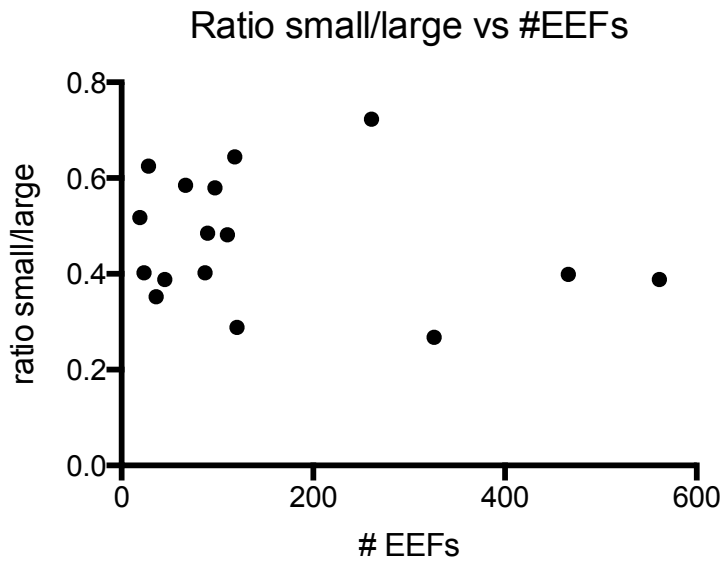


Figure S2: Ratio of hypozoite-forms/developing EEF vs total number of liver stage parasites over 16 independent assays.

Liver stage parasites were counted manually (distinguishing small and large EEF by size and number of nuclei) or with the Operetta high content screening device using the script described below in the “Supplementary protocol: Counting parasites using Operetta (Perkin-Elmer), image acquisition and analysis”. The ratio small versus large parasites was calculated for each infection and is shown here relative to the total number of parasites. The ratio small/large varies between assays, but is not correlated to the total number of EEFs.

Table S1: Transmission parameters of 53 infections.

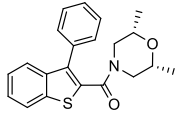
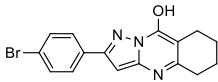
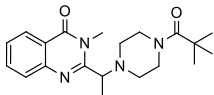
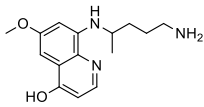
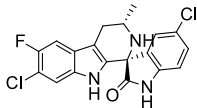
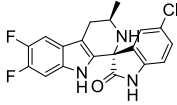
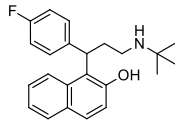
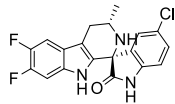
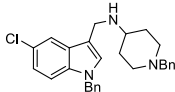
A rhesus macaque was infected with 10^6 blood stage *P. cynomolgi* parasites. Parasitemia was monitored and infected blood was taken for mosquito feedings at d 11 and 12 after infection. Oocysts were counted in 10 mosquitoes, 7 or 8 days after the infected blood meal after which a second (uninfected) blood meal was given. Sporozoites were usually harvested between 6 and 8 days after the second blood meal. ND: not determined.

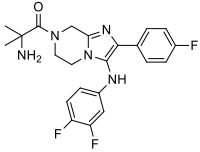
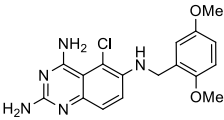
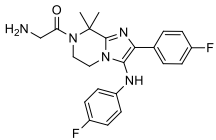
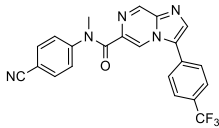
Infection nr	parasitemia @feed 1	oocyst counts feed 1	spz yield feed 1 (spz/mosq)	parasitemia @feed 2	oocyst counts feed 2	spz yield feed 2 (spz/mosq)
1	0.9	50	31000	0.9	3	nd
2	0.8	148	155000	0.3	47	44000
3	0.6	70	7500	0.7	37	0
4	0	nd	nd	0	nd	nd
5	2	248	32000	2.1	nd	23000
6	2.3	258	186000	1.5	157	147000
7	0.4	124	123000	1.3	64	66000
8	3.5	156	44000	1.9	46	58000
9	0.2	90	160000	nd	nd	nd
10	1	124	99000	1.7	44	37000
11	0.8	51	52000	0.7	26	16000
12	0.1	21	45000	0.5	27	47000
13	1.1	227	84000	0.8	83	35000
14	0.04	10	30000	0.1	14	73000
15	0.03	7	350	0.02	8	1600
16	0.48	16.8	21000	0.16	6.5	2700
17	1.76	91.2	94000	0.51	80	ND
18	2.8	89.8	69000	0.76	8.2	ND
19	0.63	200	10000	0.98	144	55000
20	0.4	43.8	37000	0.3	28.3	54000
21	1	200	77000	1.83	92.8	ND
22	0.11	55	52000	0.33	54.3	ND
23	0.65	125	116000	1.31	53	19700
24	0.9	155.4	72000	1.65	64.1	ND
25	2.2	180.8	116000	1.8	23.6	ND
26	0.19	31	44000	0.36	9.4	ND
27	1.6	182	156000	2.4	155	65000
28	2.1	179.8	106000	1.1	13.9	8000
29	1.6	151.3	33000	3.1	43.8	37000
30	0.32	32.7	ND	0.84	160.3	42000
31	0	ND	ND	0	ND	ND
32	0.03	3.4	ND	0.05	10.5	52000
33	0.0001	0.9	3600	0.0001	0.7	7700
34	1.3	200	42000	1.7	33	11000
35	2.9	95	50000	1.1	24	7500
36	0.7	106.5	128000		122	69000
37	0.13	19.9	54000	0.9	82	106000
38	0.33	24	ND	0,6	28,2	61000
39	0.8	89.1	19800	0,4	9,6	ND
40	1.27	84.4	39000	2,27	43,9	35000
41	3.6	191.2	27000	1,4	52,2	23000
42	0.9	198	13000	1,4	157	47000
43	0.43	53	ND	0,5	75,3	25000
44	2.7	146.5	62000	2,5	36	29000
45	0.02	2.6	29000	0,18	37,8	ND
46	0.43	78	73000	0,32	63	90000

47	0.75	200	68000	0,71	177	ND
48	0.5	116	37000	0,8	16,8	ND
49	1.29	40	ND	1,92	200	103000
50	0.26	144	179	0,5	107	58
51	0.46	42.6	44000	0,72	152	88000
52	2	200	111000	1,7	122	42000
53	0.05	6	7000	0,48	183	ND

Table S2: Selected Malaria box compounds evaluated in tenfold dilution series on *P. cynomolgi* liver stages.

Activity of compounds is schematically represented -: <10 % reduction, ±:10-20 % reduction, +: 20-70 % reduction, ++: 70-90 % reduction, +++:90-100 % reduction of parasite numbers at 10µM. The two compounds with the highest activity (GNF156 and KAI407, bold) were further analyzed in IC₅₀ format.

Entry	Structure	Activity against small EEF	Activity against large EEF	<i>P. yoelii</i> Liver-stage IC ₅₀ (mM)
1		-	-	not tested
2		-	-	not tested
3		-	±	0.492
4		±	-	>10
5 (KAE609)		±	±	>10
7		-	+	not tested
8		+	±	5.67
9		±	+	>10
10		+	+	1.72

11		±	++	0.277
12 (QN254)		±	+++	Not tested
13 (KAF156)		±	+++	0.001
14 (KAI407)		+++	+++	0.275

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