Supporting information to: KAI407, a potent non 8-aminoquinoline compound that kills *Plasmodium cynomolgi* dormant liver stage parasites *in vitro*

Zeeman et al.

Supporting information:

Identification of *P. cynomolgi* Hsp70 and generation of anti-PcyHsp70 antibodies.

Protocols

Operetta data acquisition and analysis

Figure S1: Correlation plots of blood stage parasiaemia in monkeys vs oocyst number in mosquitoes and oocyst number vs sporozoite yield.

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

Table S1: Transmission parameters of 53 *P. cynomolgi* infections in rhesus macaques.

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

Identification of *P. cynomolgi* Hsp70 and generation of anti-PcyHsp70 antibodies.

Based on the *P. yoelii* Hsp70 (PY06158), a search was performed in the preliminary *P. cynomolgi* genome. Alignment of the putative *P. cynomolgi* Hsp70 protein (PcyHSP70) with *P. falciparum* 3D7 Hsp70 (PfHSP70_3D7) and *P. yoelii* Hps70 (PyHSP70) is shown below. The amino acid sequence used for overexpression in *E. coli* (using a codon optimized synthetic gene) and subsequent immunization (of rabbits and mice) is indicated in bold. Upstream of the polypeptide used for overexpression, a his-tag and a thrombin tag were inserted.

```
ClustalW (v1.83) multiple sequence alignment
3 Sequences Aligned Alignment Score = nan
Gaps Inserted = 4
                                        Conserved Identities = 651
Pairwise Alignment Mode: Slow
Pairwise Alignment Parameters:
     Open Gap Penalty = 10.0 Extend Gap Penalty = 0.1
     Similarity Matrix: gonnet
Multiple Alignment Parameters:
     Open Gap Penalty = 10.0 Extend Gap Penalty = 0.1
Delay Divergent = 40% Gap Distance = 8
     Similarity Matrix: gonnet
PfHSP70_3D7 1 MASAKGSKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTER PyHSP70 1 MANAKASKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTER
PyHSP70 1 MANAKASKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTER 60
PcyHSP70 1 MASGKASKPNLPESNIAIGIDLGTTYSCVGVWRNENVDIIANDQGNRTTPSYVAFTDTER 60
PfHSP70_3D7 61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVTYQ 120
PcyHSP70 61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGIEEKPMIEVVYQ 120
PcyHSP70 61 LIGDAAKNQVARNPENTVFDAKRLIGRKFTESSVQSDMKHWPFTVKSGVDEKPMIEVSYQ 120
PfHSP70_3D7 121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN 180
PyHSP70 121 GEKKLFHPEEISSMVLQKMKENAEAFLGKSIKNAVITVPAYFNDSQRQATKDAGTIAGLN 180
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PcvHSP70
            121 GEKKLFHPEEISSMVLOKMKENAEAFLGKSIKNAVITVPAYFNDSOROATKDAGTIAGLN 180
\tt PfHSP70\_3D7 - 181 \ VMRIINEPTAAAIAYGLHKKGKGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDTHL \ 240
РУНЅР70
РСУНЅР70
              181 VMRIINEPTAAAIAYGLHKKGKGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDTHL 240
              181 VMRIINEPTAAAIAYGLHKKGKGEKNILIFDLGGGTFDVSLLTIEDGIFEVKATAGDTHL 240
{\tt PfHSP70\_3D7} \hspace{0.3cm} {\tt 241} \hspace{0.3cm} {\tt GGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEIDSL} \hspace{0.3cm} {\tt 300} \\
РУНЅР70
РСУНЅР70
              241 GGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTQCERAKRTLSSSTQATIEIDSL 300
              241 GGEDFDNRLVNFCVEDFKRKNRGKDLSKNSRALRRLRTOCERAKRTLSSSTOATIEIDSL 300
PfHSP70_3D7 301 FEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIQ 360
PyHSP70
PcyHSP70
              301 FEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIO 360
              301 FEGIDYSVTVSRARFEELCIDYFRDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIO 360
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PyHSP70 361 TLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLETAG 420
PcyHSP70 361 TLIKEFFNGKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGLETAG 420
                   ****************
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PyHSP70 421 GVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIPPA 480
PcyHSP70 421 GVMTKLIERNTTIPAKKSQIFTTYADNQPGVLIQVYEGERALTKDNNLLGKFHLDGIPPA 480
PfHSP70_3D7 481 PRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSQDEIDRMVNDAEKYKA 540
PyHSP70 481 PRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSPEEIDRMVNDAEKYKA 540
PcyHSP70 481 PRKVPQIEVTFDIDANGILNVTAVEKSTGKQNHITITNDKGRLSPEEIDRMVNDAEKYKA 540
PfHSP70 3D7 541 EDEENRKRIEARNSLENYCYGVKSSLEDQKIKEKLQPAEIETCMKTITTILEWLEKNQLA 600
PyHSP70 541 EDEENKKRIEARNSLENYCYGVKSSLEDQKIKEKLQPNEVETCMKSVTSILEWLEKNQLA 600
PcyHSP70 541 EDEENKKRIEARNSLENYCYGVKSSLEDQKIKEKLQPSEIETCMKSITTILEWLEKNQLA 600
                   PfHSP70_3D7 601 GKDEYEAKQKEAESVCAPIMSKIYQDAAGAAGGMPGGMPGGMPGGMPG-----GMN 651
*.***.**********
PfHSP70_3D7 652 FPGGMPG-AGMPGNAPAGSGPTVEEVD 677
PyHSP70 656 FPGGMPGGMGAPAGAPAGSGPTVEEVD 682
PcyHSP70 661 FPGGMPG-GGMPGGAPAGSGPTVEEVD 686
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Protocols

Generation of *P. cynomolgi* **sporozoites.** A rhesus macaque was infected with 10⁶ *P. cynomolgi M* strain blood stage parasites, thawed from a first passage stock (i.e. blood stage parasites from a monkey infected with *P. cynomolgi* M strain sporozoites). At peak parasitemia (usually days 11 and 12 post infection) 9 ml infected blood was collected into a (Lithium-)heparinised tube. The blood was kept at 38°C and fed approximately 300 three to five days old female *Anopheles stephensi* mosquitoes Sind-Kasur strain Nijmegen (Nijmegen University Medical Centre St Radboud, Department of Medical Microbiology) using a glass feeder system (1). After the second bleed, monkeys were cured by three daily intramuscular injections of 7.5 mg/kg chloroquine (2), monitored for parasite clearance 24 days after chloroquine treatment and returned to colony.

Mosquitoes were kept in climate chambers at 25°C and 80% humidity and were fed 5% D-glucose on cotton pads daily. Six or seven days after feeding on the infected rhesus blood, oocyst numbers were determined in midguts of

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 μM β-10⁻⁷M mercapto-ethanol. dexamethasone (Alfasan, Woerden, The Netherlands)).

Cells were seeded at 290,000 cells/cm² into collagen coated (5µg/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 µm 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

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 μM 4',6diamidino-2-phenylindole (DAPI) for nuclear staining. Alternatively, anti-P. cynomolgi Hsp70.1 (anti-PcyHsp70) 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-PcyHsp70 antibodies were used in 1:10,000 dilution, FITC-labeled goat-anti-rabbit IG was used as secondary antibody, 1:200 in PBS with 2µM 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-PcyETRAMP serum. PcyETRAMP 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)-IISPNDELKKEGLD and (C)-IMKHRKKERKEMED), 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 PcyETRAMP was used in 1:500 dilution, together with mouse anti-PcyHsp70 (1:10,000). As secondary antibodies TRITC-labeled anti Rabbit IgG and FITC-labeled antimouse IgG were used, in 1:200 dilution in PBS containing 2µM 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 µm 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. IC_{50} values for compounds were estimated with general least squares regression using the following equation: Percentage versus untreated = $100 * 1 / [1 + Exp((Ln IC_{50} - Ln Compound)) * Slope)]$. Where IC_{50} = 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 aqcuisition:

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 (µm²) [mean size of small EEF]

objects: large EEF

parasite size (µm²) [mean size of large EEF]

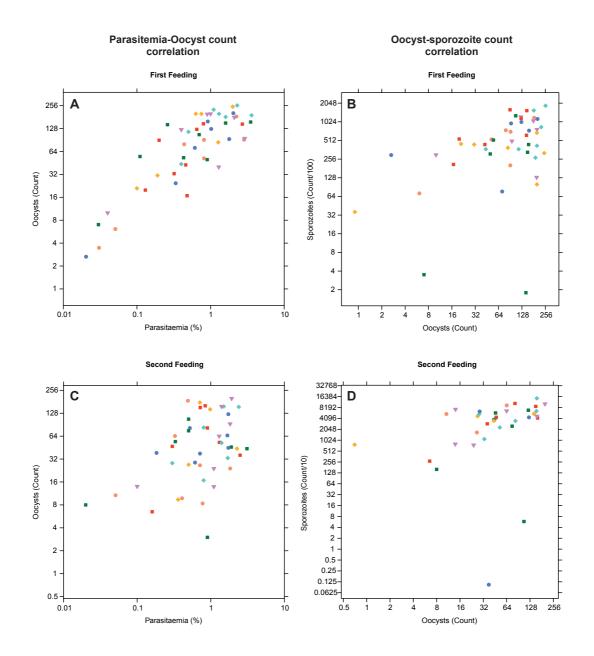


Figure S1: Correlation plots of parasitemia vs oocyst number and oocyst number vs sporozoite yield.

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

Ratio small/large vs #EEFs

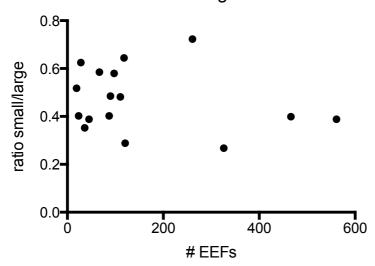


Figure S2: Ratio of hypnozoite-forms/developing EEF vs total number of liver stage parasites over 16 independent assays.

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 10⁶ 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.

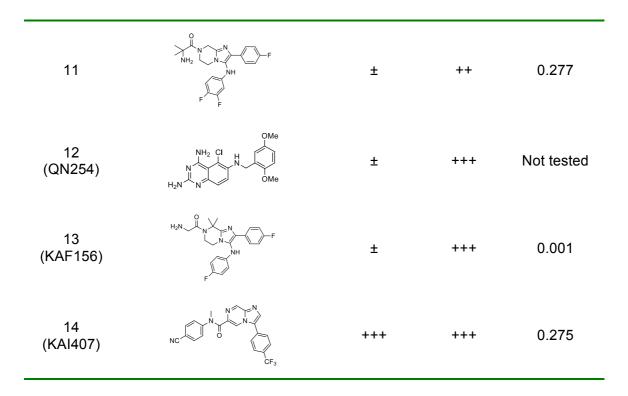
Infection nr	parasitemia @feed 1	oocyst counts feed 1	spz yield feed 1 (spz/mosq)	parasitemia @feed 2	oocyst counts feed 2	spz yield feed 2 (spz/mosq)
1	0.9	50	31000	0.9	3	nd
2	0.8	148	155000	0.3	47	44000
3	0.6	70	7500	0.7	37	0
4	0.0	nd	nd	0	nd	nd
5	2	248	32000	2.1	nd	23000
6	2.3	258	186000	1.5	157	147000
7	0.4	124	123000	1.3	64	66000
8	3.5	156	44000	1.9	46	58000
9	0.2	90	160000			nd
10	0.2 1	124		nd 1.7	nd 44	37000
10			99000			
	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	8.0	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, \pm :10-20 % reduction, \pm :20-70 % reduction, \pm :70-90 % reduction, \pm :90-100 % reduction of parasite numbers at 10 μ M. 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	P. yoelii Liver-stage IC ₅₀ (mM)
1		-	-	not tested
2	Br—N-N-N	-	-	not tested
3		-	±	0.492
4	NH ₂	±	-	>10
5 (KAE609)	F NH	±	±	>10
7	F NH NH	-	+	not tested
8	F H N N N N N N N N N N N N N N N N N N	+	±	5.67
9	F NH	±	+	>10
10	CI N NBn	+	+	1.72



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