Supplementary data

Supplementary Materials and methods

Reagents

RPMI1640 medium, glutamine, HEPES were from Euroclone; human serum was from Interstate Blood Bank, Inc.

Protino Ni-IDA resin was from Macherey-Nagel.

Synergy4 was from BioTek and Varioskan Flash multimode reader from ThermoScientific

Britelite™ was from PerkinElmer, D-luciferin powder from Stepbio, BrightGlo substrate from Promega.

Dihydroartemisinin (DHA) was either provided by Professor Richard K. Haynes (HKUST, Hong Kong) or from Sigma-Aldrich. The small libraries Malaria box and Validation set were provided by Medicine for Malaria Venture (MMV).

All other reagents and drugs were purchased from Sigma Aldrich.

P. falciparum *cultures*

The strains 3D7 and 3D7*elo1-pfs16*-CBG99 were maintained in human type 0-positive RBC at 5% hematocrit in complete medium, consisting in RPMI 1640 containing 24 mM sodium bicarbonate, with the addition of 10% (v/v) naturally-clotted heat-inactivated 0+ human serum, 0.37 mM hypoxanthine, 2 mM L-glutamine and 20 mM HEPES. The cultures were maintained at 37°C in a standard gas mixture consisting of 1-3% O_2, 5% CO_2, and 92-94% N_2.

To trigger gametocytogenesis, cultures were diluted to 0.5% parasitaemia and medium was changed daily without addition of fresh red blood cells. When culture reached more than 5%

parasitaemia, 50 mM N-acetylglucosamine (NAG) was added for 48-96 h to clear residual asexual parasites and obtain a virtually pure gametocyte culture.

Plasmid construction and parasite transfection

The integration of the *pfs16*-CBG99 luciferase cassette in the *pfelo1* locus was attempted equipping the luciferase cassette with *pfelo1* homology regions for Zinc Finger Nuclease (ZFN)-mediate genome editing (Straimer, Lee et al. 2012). Southern blot analysis of the resulting parasites with *pfelo1*- and *blasticidine-S-deaminase* (*bsd*)-specific probes on parental parasites and on parasites containing the episomal plasmids before and after transfection of the ZFN plasmids revealed however that successful disruption of the *pfelo1* locus was mediated by spontaneous integration of the entire plasmid via homologous recombination through the *pfelo1* 3' homology region

Drug handling

The standard anti-gametocyte control drugs DHA and epoxomicin were dissolved in DMSO, and diluted to the required concentration in complete medium. The final DMSO concentration was lower than 1%, which is non-toxic to the gametocytes. The drugs of the MMV Validation Set and Malaria Box were stocked in DMSO at 10 mM (room temperature for few weeks, or -80°C for long storage) or 2mM (stored at -80°C), respectively, and diluted in complete medium on the day of the experiment. Control samples were treated with DMSO at the highest concentration of treated samples.

Drug susceptibility assays

The results were expressed as the percentage viability compared with untreated controls according to the following formula: $100\times(\text{OD treated sample}- \mu_{c-})/(\mu_{c+} - \mu_{c-})$, where μ is the

mean of OD, (c+) control gametocytes and (c-) blank uninfected RBCs. To express the results of the dose response experiments, the percentage of viability was plotted as a function of drug concentration and the curve fitting was obtained by non-linear regression analysis using a four-parameter logistic method (GraphPad Prism, GraphPad Software, Inc. La Jolla, CA, USA). The IC_{50} value was extrapolated as the dose which induced a 50% inhibition of gametocytes viability (Gen5 1.10 software provided with the Synergy4-BioTek reader).

pLDH assay

Ninety-six-well plates were seeded with 200 µl per well of a parasite suspension at 2% hematocrit, 1-3% gametocytemia. After 72h incubation with the compounds, 150 µl/well were removed and the same volume of fresh complete medium was then added. Parasites were resuspended prior to use 10 µl to perform the pLDH assay (72h time point) and 90 µl to perform the luciferase assay in a black microwell plate. One hundred microliters of fresh medium were then added to the remaining parasite sample and the plate was incubated for further 72h (final hematocrit 1%), after which the pLDH assay was performed again with 20 µl of resuspended culture (72+72h time point).

MMV validation set testing with purified CBG luciferase

Luciferase in the pQE30 plasmid was expressed in *E. coli* strain BL21 as 6xHis fusion protein and whole cell extract was purified using Protino Ni-IDA resin and gravity-flow column according to the manufacturer's instructions. Protein concentration was determined with the BioRad Protein Assay system using bovine serum albumin (BSA) as the standard and purified proteins (~2 mg/0.1 L culture) were stored at 4 °C. Each compound was tested at 10 µM (final concentration) in triplicate in 384-well microplate using 10µL luciferase aliquots. Kinetic measurements were performed for 10 min (300ms integration time), after

addition of 10µL BrightGlo substrate, with Varioskan Flash multimode reader. BL emissions were normalized with respect to DMSO control and plotted using GraphPad prism (Supplementary Figure 1).

Supplementary Tables

Table S1

Primary screening (5 µM) of the MMV Validation set on early or late gametocytes from the 3D7elo1-pfs16-CBG99 transgenic strain measured by the luciferase or the pLDH assay.

MMV code	Name	LUC assay		pLDH assay	
		early GCT	late GCT	early GCT	late GCT
control drug 1	DHA	98.3	77.7	96.2	85.4
control drug 2	Epoxomicin	99.8	99.0	99.7	101.6
MMV000001	Amodiaquine	94.8	32.3	66.9	35.2
MMV000002	AQ-13	94.5	32.7	83.2	43.5
MMV000003	Artemether	98.0	82.0	73.1	79.4
MMV000004	Artenimol (Dihydro artemisinin)	98.8	85.7	77.0	84.8
MMV000005	Artesunate	98.8	88.8	76.4	96.9
MMV000006	Artemisinin	97.5	71.0	71.4	76.2
MMV000007	Azithromycin	10.7	8.2	15.7	16.8
MMV000008	Chloroquine diphosphate	94.3	33.2	79.7	31.7
MMV000009	Dapsone	0.8	6.0	-2.4	14.2
MMV000010	dehydroepiandrosterone sulphate	2.0	3.3	-3.7	15.3
MMV000011	Doxycyclin	5.3	4.5	3.0	4.7
MMV000012	Halofantrine	48.5	11.3	55.4	28.0
MMV000013	Hydroxychloroquine	94.8	32.0	58.5	36.9
MMV000014	Lumefantrine	70.5	12.3	48.3	37.8
MMV000015	Mefloquine (Racemic)	63.8	24.8	66.8	43.3
MMV000016	Mefloquine (+ RS)	63.0	22.5	72.5	54.3

MMV000017	Naphthoquine	43.7	35.7	65.5	55.8
MMV000018	NPC-1161B	24.8	47.2	43.3	62.2
MMV000019	OZ 439 mesylate	97.2	61.0	61.3	70.2
MMV000020	OZ277(RBX-11160)	97.5	65.5	68.4	68.3
MMV000021	Pamaquine (diethlyprimaquine)	18.0	37.3	42.8	52.2
MMV000022	Piperaquine phosphate	87.3	30.5	57.6	39.6
MMV000023	Primaquine	8.7	26.7	43.1	47.7
MMV000024	Pyrimethamine	-2.7	1.3	8.0	4.6
MMV000025	Pyronaridine phosphate	94.2	33.8	67.6	48.4
MMV000026	Sulfadiazine	2.0	3.8	6.4	1.5
MMV000027	Sulfamethoxazole	3.0	2.8	-2.5	9.8
MMV000028	Trimethoprim	1.2	2.2	4.1	-0.5
MMV000030	Thiostrepton	27.3	38.5	14.0	59.7
MMV000031	Cycloheximide	88.3	45.3	51.9	54.2
MMV000032	Riboflavin	3.7	5.0	5.8	9.3
MMV000033	N-acetyl-D-penicillamine	3.0	1.7	4.3	5.4
MMV000034	Deferoxamine mesylate salt	3.2	5.7	-1.3	3.1
MMV000035	Cis-Mirincamycin (HCl)	2.8	6.7	7.6	8.8
MMV000036	Trans-Mirincamycin (HCl)	5.5	7.7	16.6	5.5
MMV000037	fosmidomycin mono sodium	4.2	3.7	6.0	10.5
MMV000039	Artemisone	98.7	93.2	79.7	90.6
MMV000043	Tafenoquine	8.0	18.8	59.8	20.2
MMV000046	Atovaquone	-1.7	8.2	13.3	28.2
MMV000050	Chlorproguanil	11.5	21.3	28.9	32.0

	hydrochloride/1.11				
MMV000051	Clindamycin	4.2	1.5	2.3	6.9
MMV000053	Proguanil hydrochloride/1.14	2.3	5.2	15.8	10.2
MMV000054	Quinine sulfate dihydrate (coeff. 2)	84.2	28.5	71.2	56.8
MMV000055	Sulfadoxine	3.3	1.0	3.9	2.7
MMV000062	Pentamidine	93.3	76.8	67.0	81.7
MMV000068	Tetracycline	5.5	-1.7	9.1	-0.8
MMV000147	P218.HCl	0.0	1.0	9.1	2.3
MMV002644		67.2	16.8	54.5	39.5
MMV000061	Methylene Blue trihydrate	99.3	94.2	NA	NA

Data are expressed as % inhibition compared to untreated controls and represent the mean of three independent experiments in duplicate.

NA: Not Applicable.

Table S2.

IC_{50} (nM) on late gametocytes and transmission blocking activity of Malaria Box compounds in different gametocyte/gamete assays and in SMFA

compound ID	**LUC assay (this work)**	**pLDH assay (this work)**	Alamar Blue[3]	Imaging[4]	Sybr Green[5]	♂♀ Gamete Formation Assay[6] (wash-out mode)		Alamar Blue[7]	AO-GMT 'rounding up' assay[8]	**SMFA (direct) % reduction in oocyst intensity at 10 µM[9]**
parasite line	3D7*elo1-pfs16*-CBG99	3D7	NF54	NF54*pfs16*-GFP	NF54	♂NF54	♀NF54	NF54	3D7	NF54 p47-hsp70Luc
assay readout	luciferase reporter activity[1]	pLDH activity[2]	fluorescence	high content imaging fluorescence	fluorescence	imaging exflagellation centers	high content imaging fluorescence	fluorescence	high content imaging fluorescence	luciferase reporter activity
MMV085203	**523.3**	**235.6**	n.a.	n.a.	n.a.	331.3	inactive	7427	n.a	98,5(*)[6]
MMV019918	**539.7**	**406.9**	320	692	890	65.0	512.2	1866	825	>80
MMV000248	**605.6**	**2079.8**	310	1091	n.a.	n.a.	n.a.	1482	n.a.	~50
MMV006172	**713.8**	**732.0**	420	1364	2590	n.a.	n.a.	1482	405	100[8]
MMV019881	**752.5**	**287.6**	n.a.	n.a.	5510	n.a.	n.a.	2957	n.a.	>80
MMV665830	**811.5**	**575.7**	n.a.	541	3300	73.2	223.6	1663	2043	85[8]
MMV665941	**885.1**	**157.9**	n.a.	315	1800	419.2	445.5	5899	843	>80
MMV667491	**880.8**	**696.4**	n.a.	1060	4460	81.64	173.6	2635	1710	>80
MMV665882	**906.5**	**757.6**	n.a.	63	n.a.	n.a.	n.a.	2957	n.a.	<10
MMV666125	**942.9**	**902.2**	480	745	n.a.	n.a.	n.a.	833	n.a.	<10
MMV000448	2427.0	3136.6	1000	703	5400	247	4019	1866	643	<10
MMV019266	3414.0	2642.0	520	324	n.a.	n.a.	n.a.	11770	n.a.	<10
MMV007591	3420.9	3220.6	1150	1091	5370	n.a.	n.a.	2635	1504	100[8]

MMV011438	5115.0	6522.6	830	1130	n.a.	n.a.	n.a.	5258	n.a.	<10
MMV000442	n.d.	36.4(**)	n.a.	920	n.a.	n.a.	n.a.	332	n.a.	<10
MMV007116	n.a	41.9(**)	n.a.	253	n.a.	20742	inactive	inactive	n.a.	>80; 68,7(*)[6]
MMV020492	n.d.	1.74(**)	n.a.	n.a.	n.a.	n.a.	n.a.	inactive	n.a.	<10
MMV396797	n.d.	32.2(**)	n.a.	n.a.	8830	n.a.	n.a.	6619	2635	<10
MMV665827	n.d.	27.8(**)	n.a.	337	n.a.	16546	inactive	inactive	n.a.	>80
MMV665971	n.d.	19(**)	n.a.	228	n.a.	n.a.	n.a.	2957	n.a.	<10
MMV665980	n.d.	-6,2(**)	n.a.	n.a.	n.a.	n.a.	n.a.	11770	809	~50
MMV666021	n.d.	32.1(**)	n.a.	603	n.a.	n.a.	n.a.	inactive	n.a.	>80

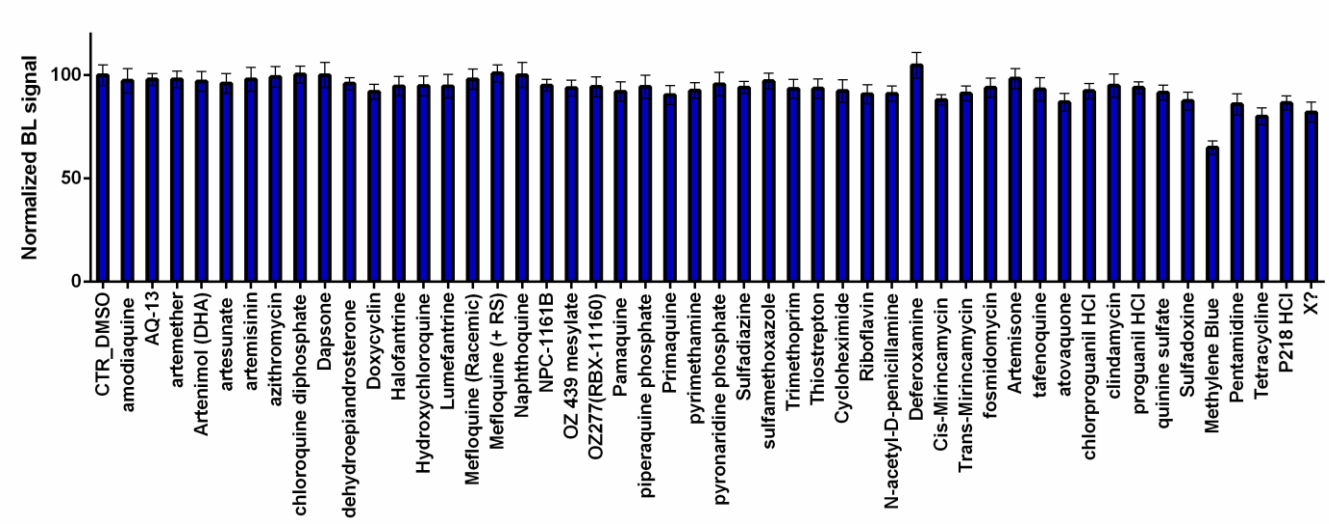
In bold are highlighted the compounds with submicroM IC_{50} values. Compounds are ranked by increasing IC_{50} value, calculated in the LUC assay.

(*) tested at 1μM, readout: oocyst number; (**): % inhibition at 3.7 μM in the pLDH primary screening on late gametocytes; n.a.: not available.

1: Cevenini L, Camarda G, Michelini E et al. Multicolor Bioluminescence Boosts Malaria Research: Quantitative Dual-Color Assay and Single-Cell Imaging in Plasmodium falciparum Parasites. *Anal Chem* 2014; **86**: 8814-21.
2: D'Alessandro S, Silvestrini F, Dechering K et al. A Plasmodium falciparum screening assay for anti-gametocyte drugs based on parasite lactate dehydrogenase detection. *J Antimicrob Chemother* 2013; **68**: 2048-58.
3: Bowman JD, Merino EF, Brooks CF et al. Antiapicoplast and gametocytocidal screening to identify the mechanisms of action of compounds within the malaria box. *Antimicrob Agents Chemother* 2014; **58**: 811-9.
4: Duffy S, Avery VM. Identification of inhibitors of Plasmodium falciparum gametocyte development. *Malar J* 2013; **12**: 408.
5: Sanders NG, Sullivan DJ, Mlambo G et al. Gametocytocidal screen identifies novel chemical classes with Plasmodium falciparum transmission blocking activity. *PLoS One* 2014; **9**: e105817
6: Ruecker A, Mathias DK, Straschil U et al. A male and female gametocyte functional viability assay to identify biologically relevant malaria transmission-blocking drugs. *Antimicrob Agents Chemother* 2014; **58**: 7292-302.
7: Sun W, Tanaka TQ, Magle CT et al. Chemical signatures and new drug targets for gametocytocidal drug development. *Sci Rep* 2014; **4**: 3743.
8: Lucantoni L, Silvestrini F, Signore M et al. A simple and predictive phenotypic High Content Imaging assay for Plasmodium falciparum mature gametocytes to identify malaria transmission blocking compounds. *Sci Rep* 2015; **5**: 16414.
9: data available at ChEMBL: http://www.ebi.ac.uk/chemblntd.

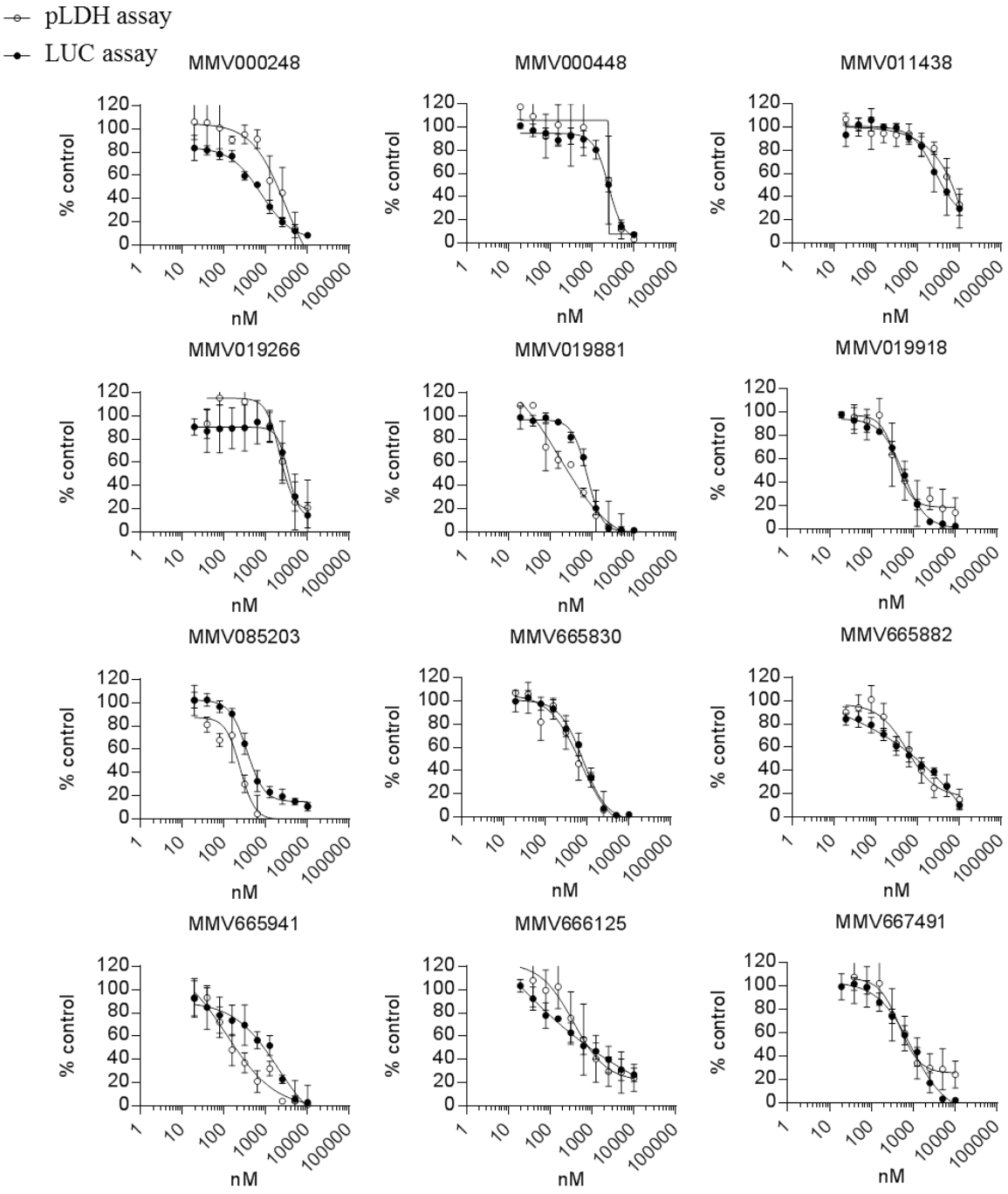
Supplementary Figures

Figure S1



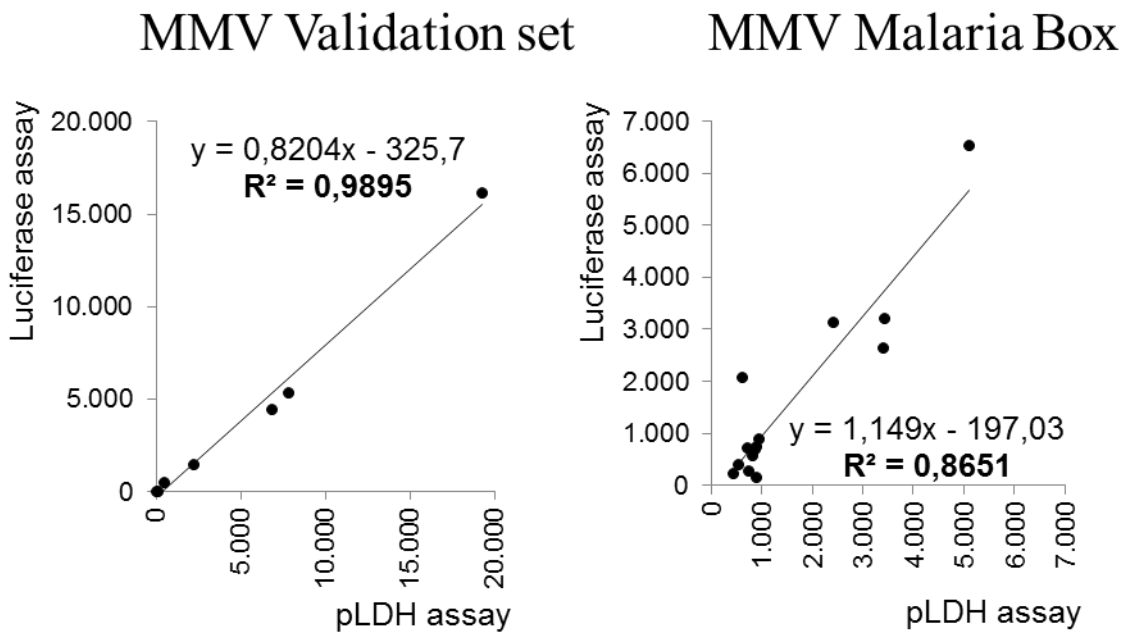
Normalized bioluminescent signal of purified recombinant CBG99 luciferase after incubation with 10 µM (final concentration) of the antimalarial drugs indicated in the *x*-axis.

Figure S2



Dose-response curves obtained by treating gametocytes with increasing doses of Malaria Box compounds and evaluating parasite viability by GC-LUC (empty circle) or pLDH assay (filled circle). The GC-LUC assay was conducted for 72 h and the pLDH assay for 72+72 h. The results expressed as percent of gametocytes compared to untreated controls. Data are the mean ± SD from at least two independent experiments in duplicate.

Figure S3



Correlation between the IC_{50} values (nM) presented in Table 2 and obtained with the GC-LUC and the pLDH assays. Compounds were selected from the MMV Validation Set (left panel) and from the MMV Malaria Box (right panel).