Supporting Information

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SI Materials and Methods

Screening Optimization. To optimize the assay conditions several parameters were varied, including the number of HepG2 cells, the number of sporozoites, type of media, media volume, and frequency of media replacement. Optimal conditions are described in *Materials and Methods* in the main text. The assay is suitable for screening as evidenced by a *Z*-factor consistently >0.5.

Western Blot Analysis of *Plasmodium* Infection. Inhibition of the liver-stage parasite was also confirmed with *Plasmodium* antibody staining of the liver cells after 44 h of infection. Infection of liver cells with *Plasmodium berghei* ANKA was performed following the procedure outlined in the *Materials and Methods*. After 44 h, cells were washed three times with PBS and then fixed with formaldyhyde. The primary antibody was 2E6 against *P. berghei* heat-shock protein 70 (1) and the secondary antibody was Alexa Fluor488-conjugated anti-mouse (Invitrogen). During the incubation with the secondary antibody the cells were also stained with Alexa Fluor 568-conjugated phalloidin (Invitrogen). Parasites and cells were visualized and quantitated with an ImageXpress Velos (Molecular Devices).

RNAi Knockdown Experiments. Knockdown experiments were done to test whether the host angiotensin and serotonin receptors are critical to *P. berghei* infection of liver cells. dsRNA in siRNA buffer (Dharmacon) was aliquoted into a pin transfer plate at 1 μM. Lipofectamine RNAiMAX (Invitrogen) was diluted 90-fold in reduced-serum Opti-MEM (Invitrogen). Using a robot arm, 1.5 μL of dsRNA was added to 9 μL of diluted Lipofectamine RNAiMAX in a 384-well tissue culture plate (Corning). After a 25-min incubation at 22 °C HepG2 cells (10,800 or 8,000 cells per well) were added to the dsRNA. After 48 h at 37 °C the cell media were exchanged and sporozoites (3,000 or 1,600 sporozoites per well, respectively) were added. After another 44 h the luciferase levels were determined with Bright-Glo (Invitrogen) and relative cell viability was determined with CellTiter-Glo (Invitrogen). Three different nontargeting siRNAs were used as negative controls (Dharmacon) and a siRNA targeting human SR-BI was used as the positive control (2, 3). SiRNAs targeting human HTR3C, HTR3D, HTR3E, AGTR2, AGTRL1, and AGTRAP were tested both pooled and unpooled, in triplicate. The experiment was repeated twice, each time under two different experimental conditions (10,800 or 8,000 cells per well) to ensure reproducibility.

In Vivo Testing of Liver-Stage Hits. In vivo tests were performed using male C57BL/6 mice, 6–8 wk old and weighing 20–24 g, purchased from Charles River and housed in the pathogen-free facilities of the Instituto de Medicina Molecular. All in vivo protocols were approved by the Animal Care Committee of the Instituto de Medicina Molecular and performed according to the regulations of the European guidelines 86/609/EEG. Salinomycin was dissolved in DMSO and then diluted 1:4 in sunflower oil. Mice received 200 μL of salinomycin solution per mouse (30 mg/kg body weight), administered orally by gavage. Control groups received an equivalent amount of vehicle. Mice were infected by i.v. injection of 1×10^4 of *P. berghei* sporozoites. Parasite load in the livers was determined 44–46 h after infection, using the in vivo Imaging System (IVIS 100 and Spectrum; Caliper Life Sciences) (4). In parallel groups, infection was allowed to proceed to the blood stage and was monitored by analysis of Giemsa-stained blood smears of tail blood collected starting at day 2 after infection.

1. Tsuji M, Mattei D, Nussenzweig RS, Eichinger D, Zavala F (1994) Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites. *Parasitol Res* 80:16–21.
2. Rodrigues CD, et al. (2008) Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection. *Cell Host Microbe* 4:271–282.
3. Yalaoui S, et al. (2008) Scavenger receptor BI boosts hepatocyte permissiveness to Plasmodium infection. *Cell Host Microbe* 4:283–292.
4. Ploemen IH, et al. (2009) Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. *PLoS ONE* 4:e7881.

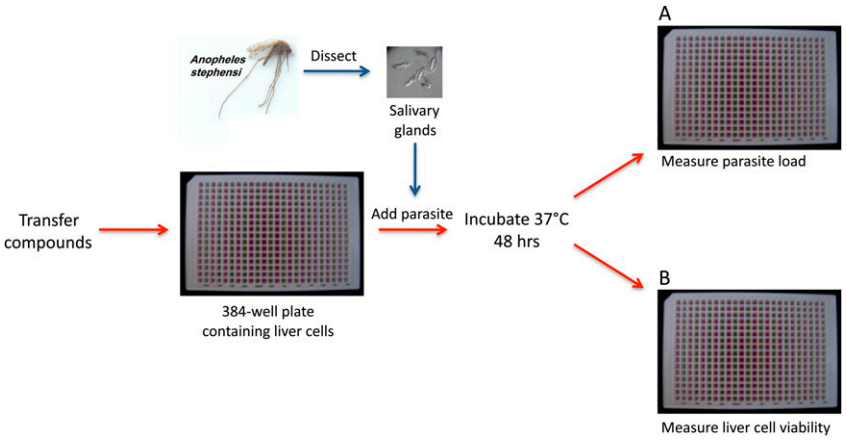

Fig. S1. Schematic of liver-stage malaria assay. Compounds and DMSO controls are added to 384-well microtiter plates containing HepG2 cells, seeded 24 h earlier in tissue culture medium. After 1 h, luciferase-expressing *P. berghei* parasites obtained from freshly dissected mosquitoes are added to the plates and the plates are incubated in a standard mammalian tissue culture incubator at 37 °C. This procedure is performed in duplicate, yielding two microtiter plates. Luminescence is measured on plate *A* to evaluate parasite load and luminescence is measured on plate *B* to evaluate mammalian cell viability.

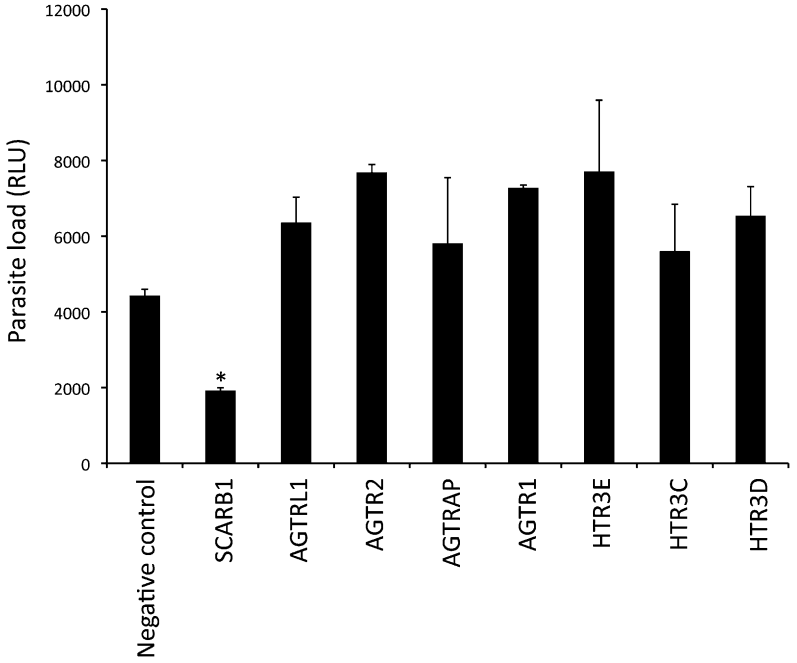

Fig. S2. RNAi knockdown of angiotensin and serotonin receptors does not affect parasite load. The expression of human angiotensin (AGTR2, AGTRL1, and AGTRAP) and serotonin (HTR3C, HTR3D, and HTR3E) receptors was knocked down before *P. berghei* sporozoite infection of HepG2 cells. Nontargeting siRNAs were used as negative controls and a siRNA targeting human SR-BI was used as the positive control. Representative data are shown as means ± SD for 8,000 cells per well infected with 1,600 sporozoites per well. *P = 0.00014 in comparison with nontargeting siRNAs, assessed by an unpaired t test.

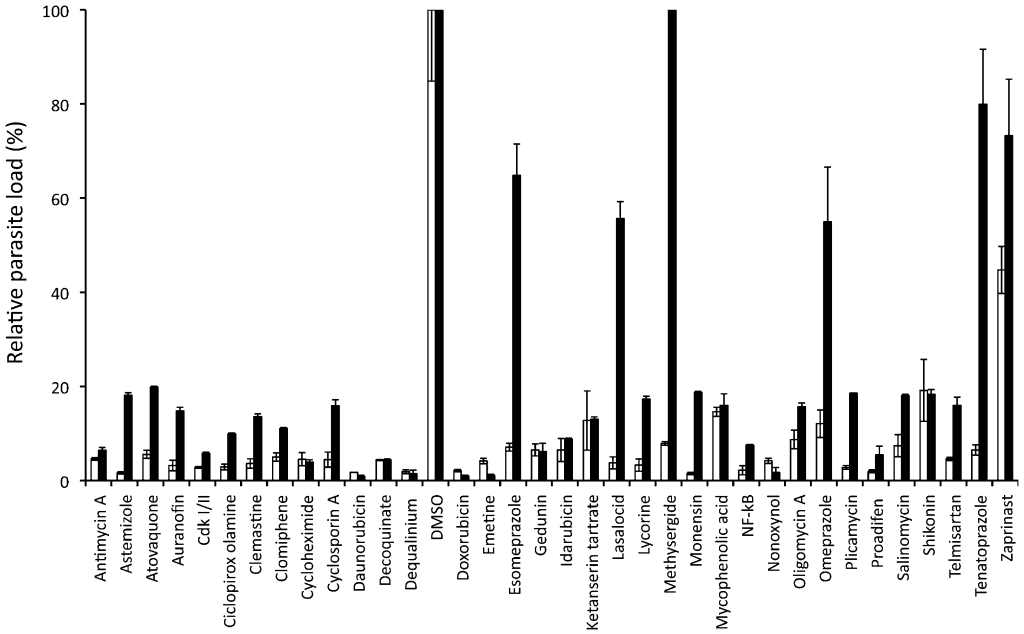

Fig. S3. Effect of HepG2 cell density on the potency of screening hits at 1 μM. HepG2 cells at 20,000 cells per well (open bars) and 5,000 cells per well (solid bars) were infected with *P. berghei* sporozoites. Esomeprazole, lasalocid, methysergide, omeprazole, and tenatoprazole are more potent at a higher density of liver cells.

Table S1. Inhibitory concentration (50%) of malaria liver screen hits

Category	Drug	HepG2 toxicity IC_{50}, μM	Liver stage IC_{50}, μM	Blood stage IC_{50}, μM	
				3D7	Dd2
Antiparasitic	Atovaquone	>10	0.0003	0.00066 (1)	
	Decoquinate	>100	0.0054	0.004 (2)	
	Emetine	10	0.080		0.018
	Lasalocid A	>100	0.011	0.200 (2)	
	Primaquine	>100	7.4		0.794
	Pyrimethamine	>10	0.0047		0.00034 (3)*
	Salinomycin	58	0.0020		0.883 (4)*
Anticancer	Daunorubicin	9.12	2.99	0.34	0.155
	Doxorubicin	11.1	0.886		0.058
	Idarubicin	12.3	6.62	>5	>5
	Plicamycin	>10	0.833	0.015	0.0125
	Shikonin	28.1	1.37	>5	>5
	Gedunin	>100	0.760		0.041 (5)*
Antibacterial/Antifungal	Ciclopirox	>100	1.05	1.2 (6)	
	Cycloheximide	>100	0.047		0.031 (7)*
	Dequalinium	>100	1.71		0.134
	Monensin	>100	0.0006		0.033 (4)*
	Oligomycin A	15	0.0005		0.111
Other	Antimycin A	>100	0.0023		0.013 (3)*
	Astemizole	12	0.114	0.227 (8)	0.457 (8)
	Auranofin	5.6	0.108	0.343	0.316
	Cdk1/2 Inhibitor III	>50	0.472		0.258
	Clemastine fumarate	>50	0.951	0.658	0.456
	Clomiphene	17	0.219		6.2 (9)
	Cyclosporin A	>50	0.0017	0.138	0.095
	Esomeprazole	>10	0.291	>50	>50
	Ketanserin	>100	1.34		>5
	Lycorine	>100	1.22		0.13 (10)*
	Methysergide	>10	0.356	>5	>5
	Mycophenolic acid	>100	1.0		>5
	NF-κB activation inhibitor	59	0.012		1.17
	Nonoxynol	61	2.31		>5
	Omeprazole	>10	0.68	>50	>50
	Proadifen	57	2.78		0.722
	Telmisartan	>10	0.025	>5	>5
	Tenatoprazole	>10	0.224	>5	>5
	Zaprinast	>10	7.79	>50	19.3

Drugs indicated in boldface type are considered selective on the basis of a 10-fold lower IC_{50} for *P. berghei* sporozoites (liver-stage) vs. *Plasmodium falciparum* blood-stage malaria.
*IC_{50} determined in various *P. falciparum* lines.

1. Baniecki ML, Wirth DF, Clardy J (2007) High-throughput Plasmodium falciparum growth assay for malaria drug discovery. *Antimicrob Agents Chemother* 51:716–723.
2. Nam TG, et al. (2011) A chemical genomic analysis of decoquinate, a plasmodium falciparum cytochrome b inhibitor. *ACS Chem Biol* 6:1214–1222.
3. Smilkstein MJ, et al. (2008) A drug-selected Plasmodium falciparum lacking the need for conventional electron transport. *Mol Biochem Parasitol* 159:64–68.
4. Yuan J, et al. (2011) Chemical genomic profiling for antimalarial therapies, response signatures, and molecular targets. *Science* 333:724–729.
5. MacKinnon S, et al. (1997) Antimalarial activity of tropical Meliaceae extracts and gedunin derivatives. *J Nat Prod* 60:336–341.
6. Weisman JL, et al. (2006) Searching for new antimalarial therapeutics amongst known drugs. *Chem Biol Drug Des* 67:409–416.
7. Ekong RM, Kirby GC, Patel G, Phillipson JD, Warhurst DC (1990) Comparison of the in vitro activities of quassinoids with activity against Plasmodium falciparum, anisomycin and some other inhibitors of eukaryotic protein synthesis. *Biochem Pharmacol* 40:297–301.
8. Chong CR, Chen X, Shi L, Liu JO, Sullivan DJ, Jr. (2006) A clinical drug library screen identifies astemizole as an antimalarial agent. *Nat Chem Biol* 2:415–416.
9. Staines HM, Dee BC, Shen MR, Ellory JC (2004) The effect of mefloquine and volume-regulated anion channel inhibitors on induced transport in Plasmodium falciparum-infected human red blood cells. *Blood Cells Mol Dis* 32:344–348.
10. Cedrón JC, Gutiérrez D, Flores N, Ravelo AG, Estévez-Braun A (2010) Synthesis and antiplasmodial activity of lycorine derivatives. *Bioorg Med Chem* 18:4694–4701.