Supplementary Materials for

A multi-stage antimalarial targets the plasmepsins IX and X essential for invasion and egress

Paco Pino[1,*], Reto Caldelari[2], Budhaditya Mukherjee[1], Juha Vahokoski[3], Natacha Klages[1], Bohumil Maco[1], Christine R. Collins[4], Michael J. Blackman[4,5], Inari Kursula[3,6], Volker Heussler[2], Mathieu Brochet[1] and Dominique Soldati-Favre[1,*]

*correspondence to: Dominique.Soldati-favre@unige.ch

This PDF file includes:

Materials and Methods

Figs. S1 to S8

Movies S1-S2

References 41 to 63

Materials and Methods

Ethics statement

All animal experiments were approved and performed in accordance with a project licence issued by the Direction générale de la santé, Domaine de l'expérimentation animale (Avenue de Beau-Séjour 24, 1206 Genève) with the authorization Number (GE/30/13) according to the guidelines set by the cantonal and international guidelines and regulations issued by the Swiss Federal Veterinary Office.

Reagents and antibodies

49c was synthetized by Actelion Pharmaceuticals as previously described (*1*). C2 was a kind gift from Dr. O. Billker (Sanger Institute, Cambridge, UK). Rapamycin was obtained from Sigma (catalogue number R0395). Stock solutions (10 μM) were prepared in DMSO and stored at −20°C. The antifolate drug WR99210 was from Jacobus Pharmaceuticals (New Jersey, USA).

Antibodies used in this study:

Polyclonal rabbit antiserum raised against the active form of PfSUB1 was used at 1/100 dilution for IFAs and 1/1000 for immunoblots (*2*).

Mouse monoclonal antibodies anti-PfMSP1 (mAb 2F10 recognises the MSP1-19 C-terminal EGF-like domains, and mAb 89.1 recognises the 83 kDa N-terminal fragment) were used for IFA and WB at 1/1000 dilution (*2*).

Polyclonal rabbit antiserum raised against recombinant full-length PfSERA5 was used at 1/10000 dilution for IFAs and immunoblots (*3*).

Two Polyclonal rabbit antisera raised against PfAMA1 were at used at 1/100 dilution for IFAs and 1/1000 for immunoblots (*4, 5*).

A series of rabbit polyclonal antisera described in the tremendous work by Crosnier et al. (*4*) was used at 1/500 dilution for IFA and WB at 1/5000 dilution (anti-PfAMA1, RH5, RON6, CyRPA, Pf113).

Anti-PfCyRPA, PfRH5 and PfRipr, have been used as described (*6*).

Anti-PfProfilin were used as described (*7, 8*).

Commercial antibodies were used according to the manufacturer recommendations:

anti-Ter-119-PerCP-Vio700 (Miltenyi Biotec), anti-HA (Clone 16B12, Covance), anti-HA (3F10, Sigma).

For immunofluorescence analysis, the secondary antibodies Alexa Fluor 488 and Alexa Fluor 594 conjugated goat α-mouse/rabbit antibodies (Molecular Probes) were used.

Cloning of DNA constructs

The transfection construct, pPM9g-Lox-PM9cDNATy3'utr-Lox-mCherry-hDHFR, used to generate the conditional knock-down of PfPMIX is based on the published plasmid pROM5g-Lox-cDNA3Ty-Lox-mCherry-HX (*9*). First, the ROM5g part was replaced a genomic fragment of PfPMIX, PMIXg used for genome integration, using KpnI and BglII, to generate pPM9g-Lox-cDNA3Ty-Lox-mCherry-HX. PfPMIXg was amplified using the primers 5'-GCGGGTACCCTCGAGCGCGTATATTAAGTGATGTAGATAAACATAGAGG-3', and 5- CGCAGATCTCATGTATGTATCTATGTGTGGTTACCATAATATC-3'.

The *Pbdhfr* 3'utr was amplified using 5'-CGCCTGCAGGGGCCCGAGGTCCATACTAACC-3' and 5'-

GCGCTGCAGAAATTGAAGGAAAAAACATCATTTGTGTTTATATG-3' on pSD279, and cloned using PstI into pPM9g-Lox-cDNA3Ty-Lox-mCherry-HX linearized with Nsi to generate pPM9g-Lox-cDNA-Ty3'utr-Lox-mCherry-HX.

A cDNA fragment of *PfpmIX* containing the last 4 exons was amplified using 5'-CCTAGGTTTTTTATTTTTTTTTTTTTATTTTTTATTTTTTTCCCAGTTTGGCACA GGAATAATACAAGGGG-3' and 5'-GGGCCCTAAATTATTTATTTTATTATGTAAGGAACTGAGATATTTTTCG-3' and cloned using ApaI and AvrII into pPM9g-Lox-cDNA-Ty3'utr-Lox-mCherry-HX to generate pPM9g-Lox-PM9cDNA-Ty3'utr-Lox-mCherry-HX. The cassette pPM9g-Lox-cDNA-Ty3'utr-Lox-mCherry was then subcloned into pPbPRF-mCherryTy-hDHFR (*7*) using XhoI and SbfI to generate the final construct pPM9g-Lox-PM9cDNATy3'utr-Lox-mCherry-hDHFR.

The PfPV1-GFP-BSD expression construct is a kind gift from Dr. J. Przyborski (University of Marburg) (*10*).

Several PasmoGEM constructs for knock-out and tagging in *P. berghei* used (*11*): PbPMIX KO (PbGEM-333835), PbPMX KO (PbGEM-342868) and PbCelTOS-HA (PbGEM-066761)

Plasmodium falciparum **parasite cultures and transfections**

P. falciparum strain 3D7 and the 3D7-DiCre parasite clone 1G5DiCre (*12*) were grown in A+ erythrocytes in RPMI-1640 medium with glutamine (Gibco), 0.2% sodium bicarbonate, 25 mM HEPES, 0.2% glucose, 5% human serum, and 0.1% Albumax II (Life Technologies). Parasite cultures were kept synchronized by double sorbitol treatments as previously described (*13*).

Late stage parasites were purified from highly synchronous cultures using Percoll (GE Healthcare) as previously described (*14*), and used for immunoblots, invasion assays, immunofluorescence assay, microscopy and transfection.

P. falciparum transfections were performed as previously described (*12*); briefly, purified mature schizonts were electroporated with 40 µg of circular plasmid DNA using the Amaxa Nucleofactor II (Lonza) and the Nucleofector™ Kits for Parasites (Lonza). Selection for transgenic parasites was performed by culture in medium containing 2.5 nM WR99210, or 2.5 mg/L blasticidin S. Selection for genome integration of transfection constructs was promoted by cycling of culture in the absence (2 weeks) and presence of WR99210. When genome integration was detected by diagnostic PCR, parasites were cloned by limiting dilution as described (*15*).

Genotyping analysis of transfectants

PCRs across *PfpmIX* were performed to confirm the transfection constructs integration, using oligonucleotides A (5'-GGCCTGCAGAAAATGTTTTTTATAAATTTTAAGAAAATAAAAAAGAAACAATTTCCG-3'), B (5'-TTATAAATTATTTATTTTATTATGTAAGGAACTGAGATATTTTTCG-3') and C (5'- GCCCGATCGTTAATCGAGCGGGTCCTGGTTCG-3'). The resulting PCR products were sequenced.

FACS analysis of malaria parasites.

Parasites (*P. falciparum* or *P. berghei*) were labelled with the DNA dye Vybrant dye cycle Green (life Technologies) for 30 min and analysed using a Beckman Coulter Gallios 4. Per sample, >50,000 cells were analysed and all measurements were performed

on independent biological replicates. For *P. berghei* gametocytes analysis, an additional purification step on Histodenz cushion was performed before labelling.

***P. falciparum* parasite egress inhibition with 49c**

Highly synchronous ring stage 3D7 or 3D7-PV1-GFP parasites were plated in triplicate at 1% parasitemia and 1% haematocrit. Cultures were treated with various doses of 49c or DMSO, at different time points and allowed to mature to schizont stage egress and reinvasion. Parasites were collected 1 hour after egress, stained with Vybrant dye cycle Green (life Technologies) and analysed by flow cytometry.

For PVM rupture assay, 3D7-PV1-GFP parasites were used. Highly synchronized cultures were treated with 49c 1 or 10 nM, or DMSO for 7-8 hours. C2 was added 2 hours before egress to synchronize egress (within a 2-5 minutes window)(*16*). When fully mature schizonts were ready to egress, the C2 was washed away with pre-warmed, pre-gassed medium (T0). Parasites were transferred to a microscopy chamber and labelled with Hoechst 33342. Images were taken by Nikon digital sight camera on a Nikon eclipse Ti inverted microscope using a 100 x oil immersion objective. Images were processed using ImageJ.

For immunoblot analysis of PfSUB1, PfAMA1, PfMSP1 and PfSERA5, synchronized cultures were prepared as described above, and a Percoll purification performed on late trophozoite stages. Purified parasites were allowed to mature and C2 was added 2 h before egress. When schizonts were fully mature, the C2 block was released (T0) and the parasites allowed to egress for 15 or 30 min. Merozoites and supernatants were lysed together without fractionation and processed with PAGE and blotting using standard

procedures.

Transmission electron microscopy

Purified parasites were prepared as for the SUB1 Western analysis; infected cells were washed with 0.1M phosphate buffer pH 7.2 and were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2, post fixed in osmium tetroxide, dehydrated in ethanol and treated with propylene oxide to embedding in Spurr's epoxy resin. Thin sections were stained with uranyl actetate and lead citrate prior to examination using a Technai 20 electron microscope (FEI Company). Samples for EM were prepared twice independently and multiple thin sections for each sample were examined.

Immuno electron microscopy (immunoEM):

Red blood cells infected with *P. falciparum* in the late schizont stage were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.05% glutaraldehyde (Electron Microscopy Sciences) in 10 mM PBS pH 7.4 for 1 h at room temperature. Cells were then pelleted and equal volume of the cell pellet was mixed with equal volume of 2% low melted agarose (Eurobio) in order of easy handling and to prevent loss of cells during subsequent extensive washing in PBS (3 × 10 min) and dehydration in graded ethanol series (50% for 15 min, 70% for 15 min, and 80% for 10 min). Cells were then infiltrated at room temperature with LR White resin (Electron Microscopy Sciences) mixed with 70% etanol (2:1) for 1 h, followed by fresh pure LR White resin for 1 h and transferred into fresh pure LR White resin for overnight at 4°C. After 2 washes in fresh LR White resin of 30 min each, cells were embedded in fresh LR White resin filled gelatine capsules ("00" size, Electron Microscopy Sciences) and polymerized at 55°C for 48 hrs.

60-nm ultrathin sections were cut with Leica Ultracut UCT microtome (Leica Microsystems) and diamond knife (DiATOME) and collected onto 2 mm single slot copper grids (Electron Microscopy Sciences) coated with 1% Pioloform plastic support film.

All steps for immunolabeling of Plasmepsin IX were performed at room temperature in the wet chamber. To block unspecific antibody binding, grids were floating on drop of blocking buffer containing 2% bovine serum albumin (BSA; AxonLab) and 0.02% Tween^{20} in 10 mM PBS for 3 × 10 min. Then grids were then incubated for 1 h with mouse anti-Ty ACIT primary antibody diluted 1: 10 or 1:50 in blocking buffer, washed for 3 × 10 min with blocking buffer and incubated with secondary goat-anti-mouse antibody coupled with 10 nm colloidal gold particles (Aurion) diluted 1:20 in blocking buffer for 1 h. Grids were washed with blocking buffer for 3 × 10 min and in PBS for 3 × 5 min. Bind antibodies were fixed to the sections with 1% aqueous glutaraldehyde for 3 min and extensively washed with double distilled water for 3 × 2 min. In control experiment, primary antibody was omitted and only secondary antibody was used.

Grids with immuno labelled sections were either post stained with 1% aqueous uranyl acetate for 10 min or directly examined using Tecnai 20 TEM (FEI) electron microscope operating at an acceleration voltage of 80 kV and equipped with a side-mounted MegaView III CCD camera (Olympus Soft-Imaging Systems) controlled by iTEM acquisition software (Olympus Soft-Imaging Systems).

Immunoblot analysis

Parasites were lysed in SDS–PAGE loading buffer under reducing conditions, subjected to two sonication cycles. SDS-PAGE was performed using standard methods. Separated

proteins were transferred to nitrocellulose membranes and probed with appropriate antibodies in 5% non-fat milk powder in 1X PBS-0.05% Tween20. Peroxidase conjugated secondary antibodies were visualized using SuperSignal (Pierce).

PfSUB1 activity assay

Fluorogenic peptide SERA4st1F-6R, based on a PfSUB1 cleavage site within its physiological protein substrate SERA4, was produced and purified as described previously (*17, 18*) by labelling N-acetylated peptide Ac-CKITAQDDEESC on both Cys side-chains with 6-iodoacetamido tetramethylrhodamine. For kinetic assays, substrate (0.2 µM) in 20 mM Tris-HCl pH 8.2, 10 mM $CaCl_2$, 25 mM CHAPS was supplemented with test compounds 49b or 49c (10 µM final concentration, diluted 1:100 from a stock in DMSO) before addition of purified recombinant PfSUB1 (~1 U/ml), prepared as described (*19*), and cleavage quantified at 21^{o}C by continuous measurement of the increase in fluorescence in a Cary Eclipse fluorimeter (Varian) fitted with a 96-well microplate reader accessory, using excitation and emission wavelengths of 552 nm and 580 nm respectively. Progress curves were displayed as individual reads from a single well of a kinetic assay; readings taken from triplicate wells in the same experiments showed no more than 2% variation from the plots shown at individual time-points.

Recombinant PfPMX and PfPMIX expression and activity assay

A codon remodeled gene encoding PMIX and PMX (Fig. S5A and B) was cloned by Gibson assembly into the pFastBacNKI-LIC-3C-2xstrepII-Flag vector (NKI Protein facility) using the primers 5'-GCTTTCAGGGACCGGGTACATGTTTTTTATTAACTTTAAAAAAATTAAAAAAA

AAC-3' and 5'-GCCCTGAAACAGCACGGTACTCAGGTTGTTAATTTTGTTATGC-3' for PMIX and 5'-GCTTTCAGGGACCCGGGTACATGAAACGCATTAGCCCG-3' and 5'-GCCCTGAAACAGCACGGTACTGTTTTTGCTTTTCGCGC-3' for PMX, respectively. The *PMX* construct was transformed to *E. coli* DH10Multibac cells (*20*) and the *PMIX* construct to MAX efficiency DH10Bac. (Invitrogen) to create the respective bacmids. The genes encoding PMIX and PMX dead mutants were generated by using Q5 site directed mutagenesis kit (NEB) using codon remodeled *PMIX* and *PMX* as templates and then back-cloned into pFastBacNKI-LIC-3C-2xstrepII-Flag vector encoding wild type PMIX and PMX using the Nde1/Blp1 and Stu1/Blp1 restriction sites, respectively. The mutated enzymes were integrated into DH10Multibac cells. Bacmids were isolated and successful integration confirmed using standard protocols (Bac-to-Bac, Invitrogen). *Sf21* cells (Gibco) were transfected using FuGene 6 (Promega) as described before (*21*). After two rounds of virus amplification, *Sf21* cells were infected with the recombinant viruses and harvested 2-4 days post-infection. Cells were resuspended in PBS supplemented with 2.5% glycerol (PBS-G), lysed with sonication, and clarified by centrifugation at 38000 g. PMIX and PMX (wild-type and dead enzymes) were captured with a Strep-Tactin or Strep-Tactin XT superflow cartridge (IBA GmbH), washed with PBS-G, and eluted with 3.5 mM D-desthiobiotin or 50 mM d-biotin. Finally, the samples were run over a size-exclusion column (Superdex 200 increase, 10/300GL, GE Healthcare Life Sciences) equilibrated with PBS-G. PMX could be purified to reasonable purity as three different sized products, which all were confirmed by peptide mass finger printing to be PfPMX. Because of low expression yields, PMIX could not be detected by

Coomassie staining and mass spectrometry but was detected in WB using anti-FLAG. The samples were concentrated and snap-frozen in liquid nitrogen.

Protease cleavage assays were performed with purified recombinant PMIX and PMX produced from insect cells(*22, 23*). PMIX and PMX cleavage assays comprised of approximately 1mg and 400 ng of PMX in digestion buffer (25 mM Tris.HCl, 25 mM MES, pH 6.4 and 5.5 for PMIX and PMX respectively; 20 mM of synthetic peptide substrate (ThermoFisher scientific, >98% purity) PfSUB1: DABCYL-G-SMLEVENDAE-G-EDANS, mutant PfSUB1: DABCYL-G-SMAAVENDAE-G-EDANS, PfAMA1: DABCYL-G-NLFSSIEIVE-G-EDANS, TgROP1: DABCYL-G-PSFVEAPVRG-G-EDANS, PfRAP1: DABCYL-G-FSSESFLENK-G-EDANS and mutant PfRAP1: DABCYL-G-FSSESFAANK-G-EDANS in 100 ul of total volume. Samples were incubated at 37°C for 5 h and processing measured as fluorescence using a SynergyH1 multi-well plate reader (BioTek) excited at 340 nm and reading emissions at 490 nm. Samples were gently shaken during incubation. Change in Relative Fluorescence Unit (RFU) was measured for each time point (0, 60, 120, 180, 240, 300 mins) by subtracting RFU from blank (without enzyme) for each time point. Inhibition of PMIX and PMX by compounds 49b (1 mM), 49c (10 nM, 100 nM) and pepstatin (10 mM) was performed in exact similar manner as mentioned above by performing the assay in the presence of each compound. The results were plotted using Prism 5.0 (GraphPad, San Diego, CA). Cleavage assay with purified recombinant PvSUB1 (200 ng) (*17*), PbCelTOS-HA ip, PfRAP1 ip and PfASP egress supernatant were performed for 8 hrs with constant shaking at 37°C. Substrates were incubated with PMIX or PMX in respective digestion buffer (50 ml total volume). In some case the assay was performed in

the presence of inhibitors Pepstatin (10 mM), 49c (100 nM) or 49b (1 mM). In all the cases the enzymes were pre incubated with the enzyme for 1 hr before adding the substrates. Samples were resuspended in SDS loading buffer and analyzed by immunoblot with rabbit a-PvSUB1 Ab, mouse a-Flag Ab, rat a-HA Ab, mouse a-PfRAP1 Ab and rabbit a-PfASP Ab .

***Plasmodium falciparum* invasion assays**

Invasion assays were performed according to Wilson *et al.* (*24*), with minor adaptations; highly synchronized parasites treated with 49c or DMSO, were purified at late trophozoite stage and cultured to schizont stage, then treated with C2 for 2 hours until full maturation. C2 was then washed away and E64 added and the merozoites released through 1.2 μm filter as described (*24*).

PfPMIX conditional knock-down phenotyping

All the excisions of *PfpmIX* were performed on synchronized culture at ring stage for 4h with either rapamycin (100 nM) or vehicle only (DMSO, 1% v/v).

Growth assay: Synchronized ring stage PfPMIX-Lox and Pf3D7-DiCre parasites were plated in triplicate at 1% parasitemia and 2.5% haematocrit. Cultures were treated with rapamycin or DMSO for 4 h washed with culture medium and cultured back for 6 days. Parasitemia was quantified every 24 h by microscopic examination on Giemsa-stained blood smears.

Intracellular development: Synchronized ring stage PfPMIX-Lox and Pf3D7-DiCre

parasites were plated in triplicate at 1% parasitemia and 2.5% haematocrit. Cultures were treated with rapamycin or DMSO for 4 h washed with culture medium and cultured back. Images were taken 5, 20 and 44 h post invasion.

Re-invasion assay: As above, synchronized ring stage PfPMIX-Lox and Pf3D7-DiCre parasites were plated in triplicate at 1% parasitemia and 2.5% haematocrit. Cultures were treated with rapamycin for 4 h and allowed to mature till egress. 2 h after egress parasites were collected and treated with trypsin or not for 4 min. at 37°C (Gibco 15090), washed with 2% (w/v) BSA in PBS, and stained with Vybrant dye cycle Green for FACS analysis.

PfRAP1 processing analysis: *PfpmIX* was induced at ring stage as described above. In addition, parental PfPMIX-Lox strain was treated with 49c for 8 hours before egress. Fully mature schizonts were collected and processed for immunoblots analysis.

Immunofluorescences (IFA)

IFAs were performed according to described procedures (*25*). Fully mature schizonts were smeared onto glass slides and fixed with 4% paraformaldehyde with 0.0075% glutaraldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.1% (v/v) Tx100 in PBS at room temperature for 10 min, washed twice for 5 min in PBS, and blocked over-night at 4°C with 3% (w/v) BSA in PBS. All primary antibodies were used at 1/100 dilution in PBS/0.5% (v/v) Tween 20/1% (w/v) BSA for 1 hour at room temperature. Secondary antibodies Alexa Fluor 488 and Alexa Fluor 594 conjugated goat α-mouse/rabbit antibodies (Molecular Probes) were used

Finally, parasites were counter-stain with 1 µg/mL DAPI in PBS. Confocal images were taken with a Zeiss laser scanning confocal microscope (LSM700, objective apochromat 63x /1.4 oil) at the bio-imaging facility of the Faculty of Medicine, University of Geneva. Image stacks were processed with ImageJ using maximum intensity Z projection.

Immunoprecipitations

Highly synchronized ring stage parasites were treated with were treated with rapamycin or DMSO for 4 h washed with culture medium and cultured back. Resulting trophozoites parasites were Percoll-purified and treated with C2 for 2 hours. When schizonts were fully mature, the C2 block was released and the parasites allowed to egress 30 min in protein free RPMI medium, at 37°C under Trigaz conditions. Free merozoites and supernatants were collected by centrifugation at 600g for 10 minutes. Co-immunoprecipitations were performed as previously described (*26*). Free merozoites were lysed in TNET (1% TX100, 150 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.4) plus protease inhibitors (Roche) with three cycles liquid N_2/37°C, and too sonication cycles. Soluble protein extracts and egress supernatants were incubated with rabbit anti-PfCyRPA serum for 2 hours at 4°C with rotation. 100 µL of PBS-washed magnetics Dynabeads-Protein G were added for 2 hours at 4°C with rotation according to the manufacturer.

Time-Lapse Microscopy

P. falciparum egress was imaged as described (*16*), using C2 to synchronize egress. Microscopic DIC images were collected at 5 s intervals for up to 30 min. Image files were exported as AVI movies using Axiovision 3.1 software. Time to individual egress

events was recorded by visual examination of movie frames.

P. berghei parasite maintenance and culture

P. berghei ANKA strain 2.34, 820cl1 (*27*) and derived transgenic lines were maintained in female CD1 outbred mice (Charles River, France).

To generate a parasite line expressing CelTOS-3xHA, PlasmoGEM vector pbGEM-066761 was transfected in the 820cl line.

Development of intra-erythrocytic parasites and parasitemia were monitored on Giemsa-stained blood smears every day.

49c was used *in vivo* at 100 mg/kg, injected ip, and suspended in 70% Tween-80 (d= 1.08g/ml) and 30% ethanol (d=0.81g/ml), followed by a 10-fold dilution in H_2O as previously described (*1*).

P. berghei exflagellation assay

Gametocytes were isolated from 49c-treated or control CD1 mice three days after infection. 4 µl of tail blood were mixed with 70 µl exflagellation medium (RPMI 1640, 25 mM HEPES, 4 mM sodium bicarbonate, 5% FCS, 100 µM xanthurenic acid, pH 7.4). The number of exflagellation centres and red blood cells (RBCs) was counted in a haemocytometer at 20 minutes post-activation. Microgametocytemia was quantified on Giemsa-stained smears to calculate the number of exflagellation centres per 100 microgametocytes.

Gametocytes purification:

P. berghei gametocytes egress assay

Gametocytes were collected and purified from 49c-treated or control phenyl hydrazine-treated mice. Blood was collected with intracardiac puncture and immediately mixed with suspended animation (SA - RPMI1640, 25 mM HEPES, 5% FCS, 4 mM sodium bicarbonate, pH 7.20) to prevent gametocytes activation. Gametocytes were separated from uninfected RBCs on a Histodenz cushion made up from 48% of a Histodenz stock (27.6% w/v Histodenz in 5.0 mM Tris-HCl [pH 7.20], 3.0 mM KCl, 0.3 mM EDTA) and 52% SA with a final pH of 7.2. Gametocytes were harvested from the interphase.

49c was used at 1 nM for *in vitro* treatments.

Gametocytes were activated by addition of 20 volumes of exflagellation medium at T0 and allowed to egress for 5 or 10 minutes. Samples where quickly chilled by addition of ice cold PBS. Gametocytes were then stained with anti-Ter119 antibodies according to the manufacturer procedure, and analysed by FACS.

P. berghei ookinetes cultures

PbCelTOS-HA parasites were maintained in phenyl hydrazine-treated mice. Ookinetes were produced *in vitro* by adding 1 volume of high gametocyteamia blood into 20 volumes of ookinete medium (RPMI1640, 25 mM HEPES, 10% FCS, 100 µM xanthurenic acid, pH 7.4) and incubated at 19°C for 20-24 h. Conversion and ookinetes maturation was evaluated on Giemsa-stained blood smears as previously described (*28*).

Ookinetes were purified using anti-p28 mouse monoclonal antibody coupled with magnetic beads (*28*) and analysed by immunoblots using anti-HA antibodies to visualize

PbCelTOS processing and anti-tubulin as loading control.

P. berghei parasites used for hepatic and mosquito stages

Selection marker-free *P. berghei* ANKA expressing mCherry under the control of hsp70 regulatory sequences (PbmCherry$_{hsp70}$)(*29*), and *P. berghei* ANKA expressing mCherry under Hsp70 together with Firefly luciferase under ef1a promoter (PbmCherry$_{hsp70}$+Luc $_{ef1a}$)(*30*).

Detached cell formation: Confluent HeLa cell cultures (40'000 cells/96well seeded the day before) and HepG2 cell cultures (60'000 cells/96well seeded the day before) were infected with 20'000 *P. berghei* parasites for 2 hours. The cells were detached by the use of accutase (Innovative Cell Technology), were pooled and the equivalent of each well was distributed to 8 new wells containing drug or control. Medium was changed at 24hpi and 48h. At 48hpi, parasite sizes and numbers were determined using an automated microscope (IN Cell analyzer, GE lifesciences) monitoring the mCherry signal of PbmCherry$_{hsp70}$ parasites (see Fig. S8). At 65hpi the detached cells in the medium supernatants were determined by fluorescent microscopy (Leica DMI 6000B). The detached cell formation rate (48 hours parasite numbers = 100%)) is presented graphically with medians and interquartile range. Statistical analysis was done using Prism (GraphPad) by performing One-way ANOVA with Dunnet's Multiple Comparisons (* $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$, ns/not significant > 0.05)

In vivo imaging: Balb/C mice were infected with 50'000 PbmCherry$_{hsp70}$+Luc $_{ef1a}$ sporozoites by i.v. injection. At 20hpi and 40hpi mice were either treated with 49c (100 mg/kg) or with solvent control. At indicated time points mice were anaesthetized with Isoflurane. 100ul Rediject-D-luciferin (30mg/ml; PerkinElmer) was i.p. injected and

exactly 10 minutes later measured for 3 minutes under anaesthesia in the IVIS Lumina II imager (PerkinElmer/Caliper). The image quantification was done with the machine's Living Image 4.4 software. The pictures show the radiance with settings from 3e5 to 3e7 (photons/sec/cm^2/sr). The graph is depicting luminescence as total flux (photons/second) of the region of interest. A ROI representing the head and chest area was chosen to monitor luminescent signal in the circulating blood. From each individual mouse tail vein blood was collected and parasitemia was evaluated by Novocyte flow cytometer (Acea Biosciences) monitoring parasite's mCherry signal.

Immunofluorescent analysis: HeLa cells grown on cover slips (Marienfeld) were infected with $PbmCherry_{hsp70}$ sporozoites. At 54hpi and 65hpi cells were fixed with 4%PFA for 10 min at Room temperature, permeabilized with cold 100% Methanol for 10' at -20°C, blocked with 10% FCS in PBS. Rat anti-Msp1 and chicken anti-Exp1 antibodies were used as primary antibodies in 10% FCS/PBS. These were detected with anti- rat AlexaFluor488 (Thermofisher) and anti-chicken Cy5 (Jackson Immuno Research) in 10%FCS/PBS. DNA is visualized by Hoechst 33342 (Thermofisher). The stained cells were mounted with Dako Fluorescent Mounting medium on microscope slides. Confocal imaging pictures were taken with a Leica TSC SP8 microscope (63x oil objective, PMT and HyD detectors).

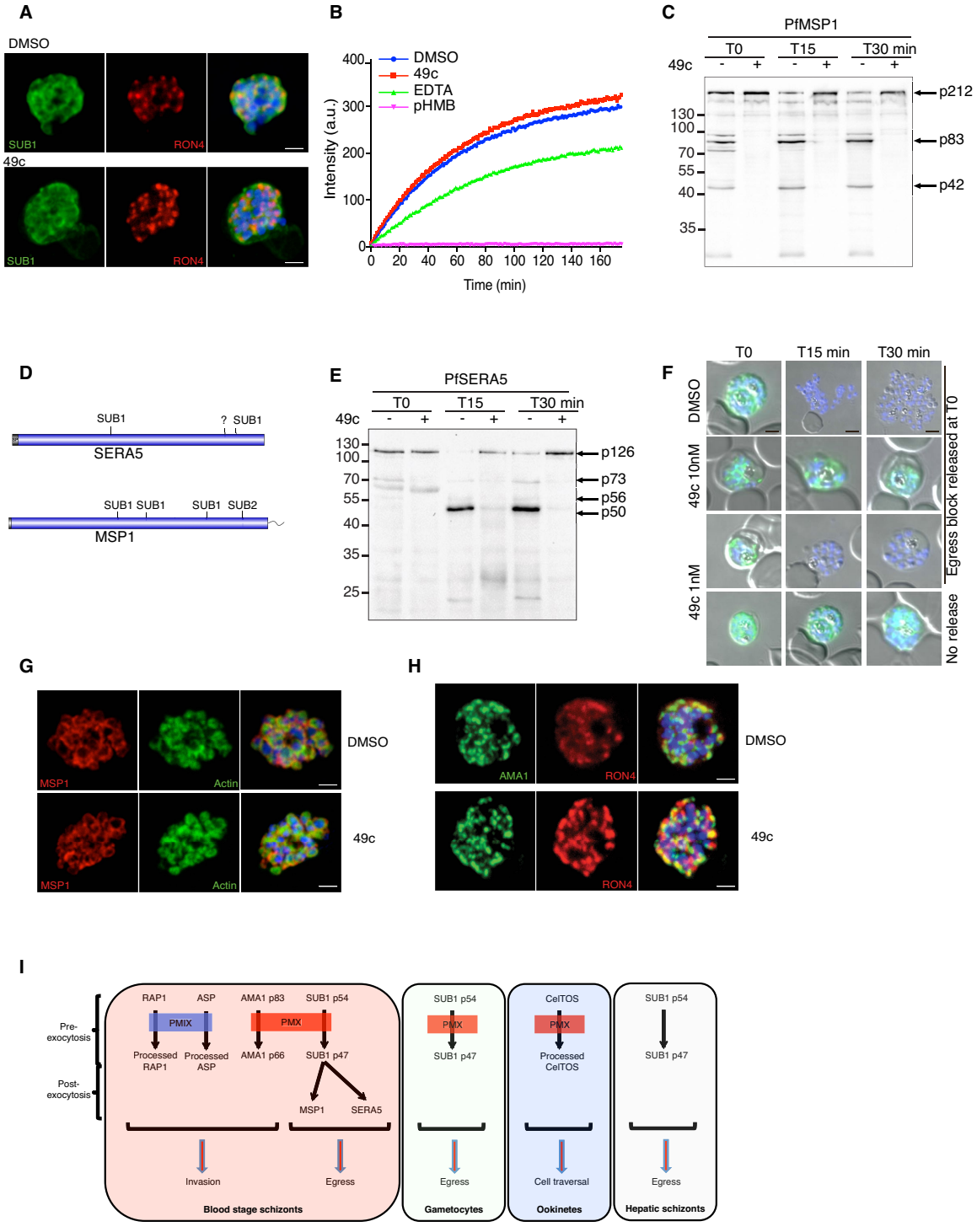
Liver stage evaluation: confluent HeLa cells (40000 cells/96well seeded the day before) were infected with 20'000 $PbmCherry_{hsp70}$ sporozoites for 2h. The cells were detached by the use of accutase (Innovative Cell Technology), pooled and the equivalent of each well was distributed to 8 new wells containing drug (CELLVIS-96well glass bottom plate) or control. Media were changed at 24 hpi and 48 hpi. At 54 and 65 hpi medium was

collected (for detached cell counting) and adherent cells were fixed with 4%PFA for 10 minutes at room temperature. Cells were then permeabilized, blocked with 10%FCS in PBS. Staining of fixed cells was done with rat anti-Msp1 polyclonal antibody and subsequently anti-rat Alexa488. Hoechst was used as a DNA counterstain. Classifications after Burda et al. (*29*): schizont stage is either negative for MSP1 or show MSP1 staining solely at the parasite plasma membrane (without invaginations). Cytomere stage shows plasma membrane staining of Msp1 with clear invaginations (see Fig. 4c). At merozoite stage the MSP1 is seen at the plasma membrane of individual merozoites. The different classes are presented as percentages with mean an SD of 3 independent experiments.

Mosquito transmission experiment:

Balb/C mice were infected with PbmCherry$_{hsp70}$+Luc $_{ef1a}$. At 10% parasitemia mice were drug or control treated (100 mg/kg i.p.) for 30 h. From each mouse 100-150 mosquitoes were allowed to feed. Unfed mosquitoes were removed after 1 day. At day 7 midguts (n=15) were dissected into 5µl drops of phenol-red free Williams' Medium E on 21-welled diagnostic microscope slides (Menzel-Gläser). To avoid disintegration of the oocysts only 15 mosquitoes were processed at a time. After covering with cover glass fluorescent and phase contrast pictures were taken as immediate as possible on Leica DM6000B with 5x objective. The fluorescent oocysts were counted from pictures by the help of the "find maxima" (noise tolerance 2) in ImageJ.

Figure S1



Figures S1:

A) IFAs showing the localisation of PfSUB1, relative to the rhoptry protein PfRON4 under 49c treatment. **B)** Typical progress curves showing cleavage *in vitro* of fluorogenic PfSUB1 substrate SERA4st1F-6R by recombinant PfSUB1 in the presence of 2% (v/v) DMSO only (vehicle control), 49c (10 μM), EDTA (10 mM) or p-hydroxymercuribenzoate (pHMB, inhibitor control, 1 mM). Neither of the test compounds had any inhibitory effect on PfSUB1 catalytic activity at these concentrations. **C)** Immunoblots evaluating the processing of PfMSP1 upon treatment with DMSO/49c. *P. falciparum* blood stage parasites were treated with C2 ± 49c (1 nM) for 6 h. When fully mature, the C2 block was released and the parasites allowed to egress for 15 or 30 min. Merozoites and supernatants were lysed together without fractionation. P212 precursor and p83 and p42 cleave products are indicated with arrows. **D)** Schematic of PfSERA5 and PfMSP1 maturation steps; the cleavage sites and the proteases responsible are indicated when known. **E)** Immunoblots evaluating the processing of PfSERA5 upon treatment with DMSO/49c. *P. falciparum* blood stage parasites were treated with C2 ± 49c (1 nM) for 6 h. When fully mature, the C2 block was released and the parasites allowed to egress for 15 or 30 min. Merozoites and supernatants were lysed together without fractionation. P126 precursor and p73, p56 and p50 cleave products are indicated with arrows. **F)** *P. falciparum* parasites expressing a PV1- GFP fusion were treated with C2 ± 49c (1 or 10 nM). When fully mature, the C2 block was released and the parasites allowed egressing. The disappearance of the PV1-GFP signal was used as indicator for the PV rupture. Scale bar: 2 μm. **G)** IFAs showing the localisation of PfMSP1, relative to the cytosolic protein PfActin under 49c treatment. **H)** IFAs showing the localisation of PfAMA1, relative to the rhoptry protein PfRON4

under 49c treatment. **I)** Scheme showing the substrates of PMIX and PMX in the different life stages of the parasite.

Figure S2

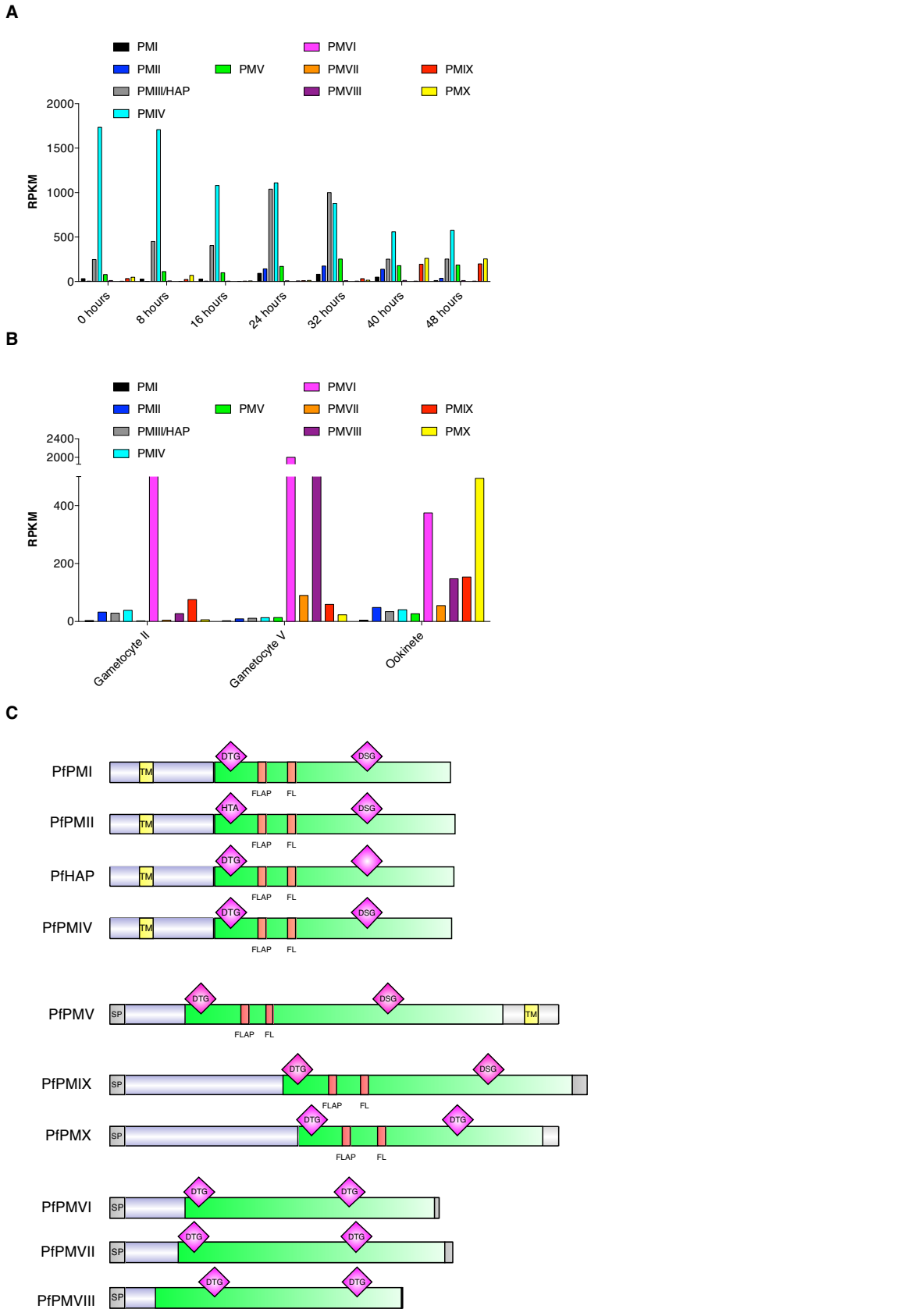

A
PMI
PMII
PMIII/HAP
PMIV
PMV
PMVI
PMVII
PMVIII
PMIX
PMX
RPKM
2000
1500
1000
500
0
0 hours
8 hours
16 hours
24 hours
32 hours
40 hours
48 hours
B
PMI
PMII
PMIII/HAP
PMIV
PMV
PMVI
PMVII
PMVIII
PMIX
PMX
RPKM
2400
2000
400
200
0
Gametocyte II
Gametocyte V
Ookinete
C
PfPMI
PfPMII
PfHAP
PfPMIV
PfPMV
PfPMIX
PfPMX
PfPMVI
PfPMVII
PfPMVIII
DTG
HTA
DSG
TM
SP
FLAP
FL

Figure S2:

A and B) Expression profiles of plasmepsins based on RNAseq data (http://plasmodb.org), in asexual blood stages (A) and gametocytes and ookinetes (B); **C)** Repertoire of *P. falciparum* plasmepsins. Schematic of the structure of the 10 plasmepsins encoded in the *P. falciparum* genome. Putative or known prodomains are in grey, transmembrane domains in yellow, mature catalytic conserved domains are in green, active motive (DTG, DSG) in pink, FLAP and flexible loop regions are in red.

Figure S3

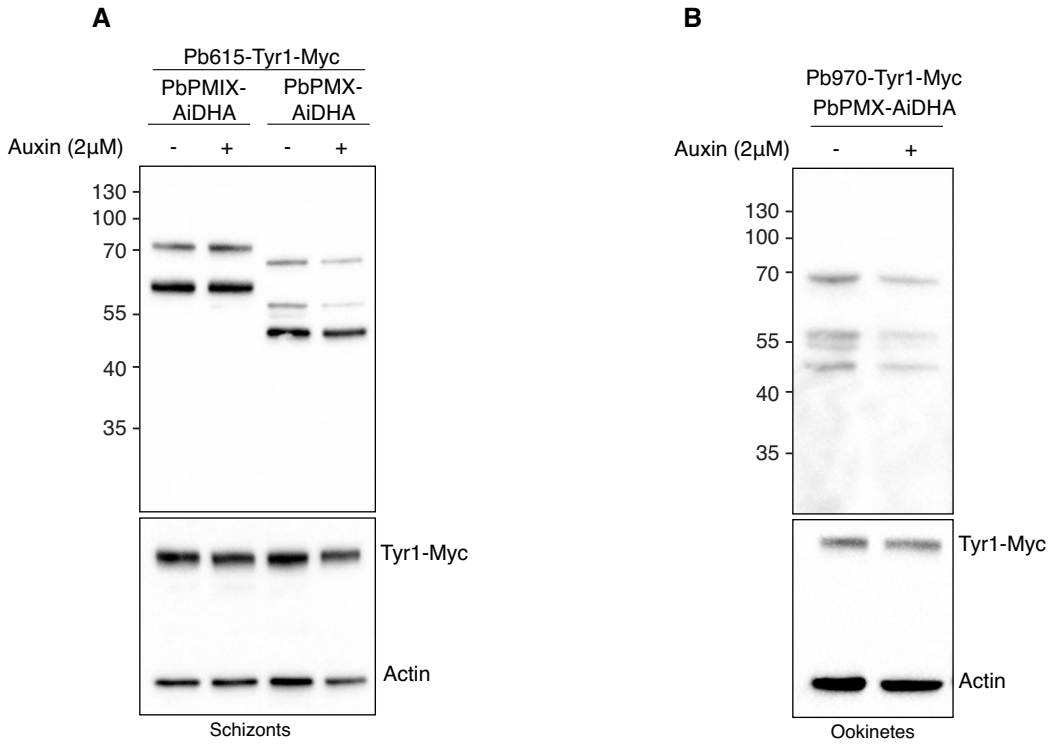


Figure S3:

A) Immunoblot showing the expression of PbPMIX-AiD-HA and PbPMX-AiD-HA in the Pb615-Tyr1-Myc parental line. Schizonts were maturated *in vitro* in the presence or absence of 2 µM auxin and processed for immunoblot analysis. **B)** The same experiment was performed for PbPMX in the Pb970-Tyir-Myc parental line.

Figure S4

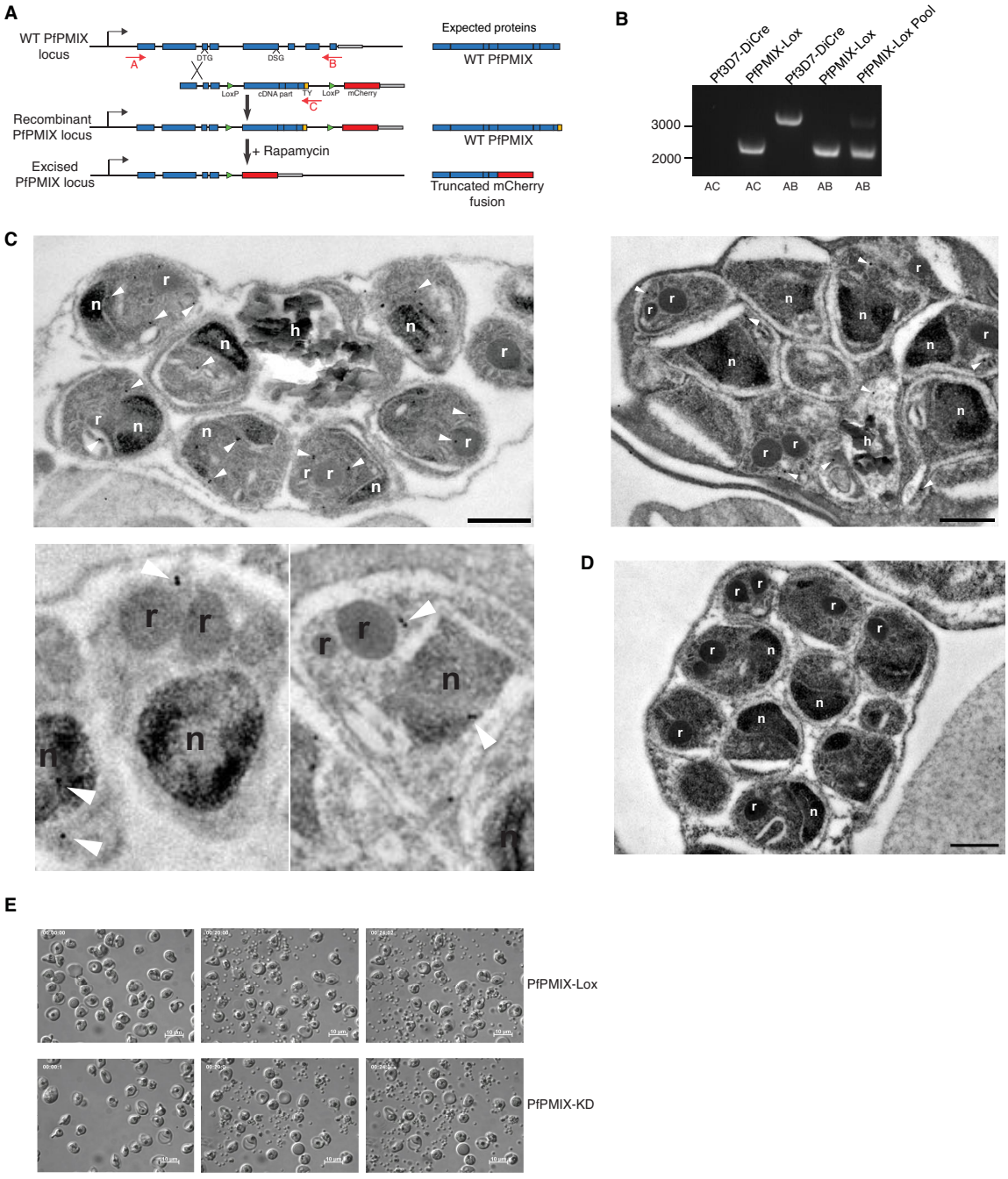


Figure S4:

A) Scheme of the strategy used to generate the PfPMIX inducible excision strain. The first loxP site is inserted in the third intron, a Ty epitope tag at the end of the CDS and a second loxP site after its 3'UTR. A mCherry epitope tag was added after the second loxP

site. The primers used for genotyping are shown in red. Upon activation of the DiCre recombinase activity by addition of rapamycin the last four exons of PfPMIX are excised producing a truncated inactive mCherry fusion protein lacking the half of the catalytic motive. **B)** Agarose gel of genotyping PCR reactions performed on genomic DNA extracted from a PfPMIX-Lox parasite clone and parental 3D7-DiCre. **C)** Post-embedding immune labeling of PMIX in schizont stage of *P. falciparum* infected red blood cells. *P. falciparum* infected red blood cells were processed for immune electron microscopy and embedded in LR White resin. Localization of the gold particles is highlighted with white arrowheads, predominantly in the secretory pathway with endoplasmic reticulum staining and accumulation around the rhoptries suggesting again a pre-rhoptry organelle localization. Rhoptries (r) and nucleus (n). h: haemozoin crystals; scale bars: 0.5 mm. **E)** Snapshots from video microscopy movies. Highly synchronized PfPMIX-Lox and PfPMIX-KD parasites culture were treated with rapamycin and C2. When fully mature, the C2 block was released and the schizonts monitored for egress.

Figure S5

A

>Codon remodelled PfPMX

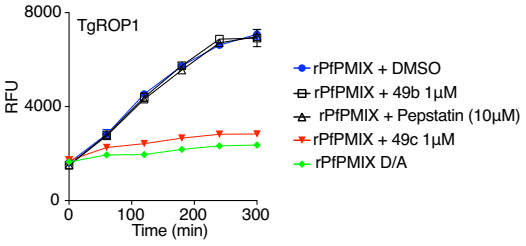
ATGAAACGCATTAGCCCGCTGAACACCCTGTTTTATCTGAGCCTGTTTTTTAGCTATACCTTTAAAGGCCTGAAATGCACCCGCATTTATAAAATTGGCACCAAAGCGCTGCCGTGCAGCGAATGCCATGATGTG
TTTGATTGCACCGGCTGCCTGTTTGAAGAAAAAGAAAGCAGCCATGTGATTCCGCTGAAACTGAACAAAAAAAACCCGAACGATCATAAAAAACTGCAGAAACATCATGAAAGCCTGAAACTGGGCGATGTGA
AATATTATGTGAACCGCGGCGAAGGCATTAGCGGCAGCCTGGGCACCAGCAGCGGCAACACCCTGGATGATATGGATCTGATTAACGAAGAAATTAACAAAAAACGCACCAACGCGCAGCTGGATGAAAAAAA
CTTTCTGGATTTTACCACCTATAACAAAAACAAAGCGCAGGATATTAGCGATCATCTGAGCGATATTCAGAAACATGTGTATGAACAGGATGCGCAGAAAGGCAACAAAACTTTACCAACAACGAAAACAACAG
CGATAACGAAAACAACAGCGATAACGAAAACAACAGCGATAACGAAAACAACCTGGATAACGAAAACAACCTGGATAACGAAAACAACAGCGATAACAGCAGCATTGAAAAAAACTTTATTGCGCTGGAAAACA
AAAACGCGACCGTGGAACAGACCAAAGAAAACATTTTTCTGGTGCCGCTGAAACATCTGCGCGATAGCCAGTTTGTGGGCGAACTGCTGGTGGGCACCCCGCCGCAGACCGTGTATCCGATTTTTGATACCG
GCAGCACCAACGTGTGGGTGGTGACCACCGCGTGCGAAGAAGAAAGCTGCAAAAAAGTGCGCCGCTATGATCCGAACAAAAGCAAAACCTTTCGCCGCAGCTTTATTGAAAAAAACCTGCATATTGTGTTTG
GCAGCGGCAGCATTAGCGGCAGCGTGGGCACCGATACCTTTATGCTGGGCAAACATCTGGTGCGCAACCAGACCTTTGGCCTGGTGGAAAGCGAAAGCAACAACAACAAAAACGGCGGCGATAACATTTTT
GATTATATTAGCTTTGAAGGCATTGTGGGCCTGGGCTTTCCGGGCATGCTGAGCGCGGGCAACATTCCGTTTTTTGATAACCTGCTGAAACAGAACCCGAACGTGGATCCGCAGTTTAGCTTTTATATTAGCCC
GTATGATGGCAAAAGCACCCTGATTATTGGCGGCATTAGCAAAAGCTTTTATGAAGGCGATATTTATATGCTGCCGGTGCTGAAAGAAAGCTATTGGGAAGTGAAACTGGATGAACTGTATATTGGCAAAGAACG
CATTTGCTGCGATGAAGAAAGCTATGTGATTTTTGATACCGGCACCAGCTATAACACCATGCCGAGCAGCCAGATGAAAACCTTTCTGAACCTGATTCATAGCACCGCGTGCACCGAACAGAACTATAAAGATAT
TCTGAAAAGCTATCCGATTATTAAATATGTGTTTGGCGAACTGATTATTGAACTGCATCCGGAAGAATATATGATTCTGAACGATGATGTGTGCATGCCGGCGTATATGCAGATTGATGTGCCGAGCGAACGCAAC
CATGCGTATCTGCTGGGCAGCCTGAGCTTTATGCGCAACTTTTTTACCGTGTTTGTGCGCGGCACCGAAAGCCGCCCGAGCATGGTGGGCGTGGCGCGCGCGAAAAGCAAAAACTAA

B

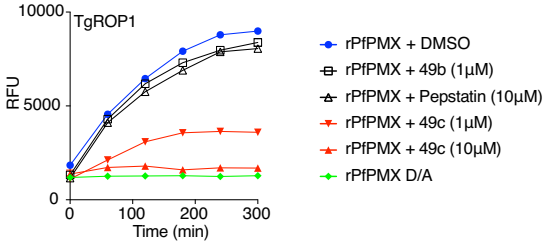
>Codon remodelled PfPMIX

ATGTTTTTTATTAACTTTAAAAAAATTAAAAAAAAACAGTTTCCGATTTATCTGACCCAGCATCGCATTATTACCGTGTTTCTGATTTTTATTTATTTTATTAACCTGAAAGATTGCTTTCATATTAACAACAGCCGCATT
CTGAGCGATGTGGATAAACATCGCGGCCTGTATTATAACATTCCGAAATGCAACGTGTGCCATAAATGCAGCATTTGCACCCATGAAAACGGCGAAGCGCAGAACGTGATTCCGATGGTGGCGATTCCGAGCA
AACGCAAACATATTCAGGATATTAACAAAGAACGCGAAGAAAACAAATATCCGCTGCATATTTTTGAAGAAAAAGATATTTATAACAACAAAGATAACGTGGTGAAAAAAGAAGATATTTATAAACTGCGCAAAAAAA
AAAAACAGAAAAAAAACTGCCTGAACTTTCTGGAAAAAGATACCATGTTTCTGAGCCCGAGCCATGATAAAGAAACCTTTCATATTAACCATATGAACAAAATTAAAGATGAAAAATATAAACAGGAATATGAAGAA
GAAAAAGAAATTTATGATAACACCAACACCAGCCAGGAAAAAAACGAAACCAACAACGAACAGAACCTGAACATTAACCTGATTAACAACGATAAAGTGACCCTGCCGCTGCAGCAGCTGGAAGATAGCCAGTA
TGTGGGCTATATTCAGATTGGCACCCCGCCGCAGACCATTCGCCCGATTTTTGATACCGGCAGCACCAACATTTGGATTGTGAGCACCAAATGCAAAGATGAAACCTGCCTGAAAGTGCATCGCTATAACCATA
AACTGAGCAGCAGCTTTAAATATTATGAACCGCATACCAACCTGGATATTATGTTTGGCACCGGCATTATTCAGGGCGTGATTGGCGTGGAAACCTTTAAAATTGGCCCGTTTGAAATTAAAAACCAGAGCTTTG
GCCTGGTGAAACGCGAAAAAGCGAGCGATAACAAAAGCAACGTGTTTGAACGCATTAACTTTGAAGGCATTGTGGGCCTGGCGTTTCCGGAAATGCTGAGCACCGGCAAAAGCACCCTGTATGAAAACCTGA
TGAGCAGCTATAAACTGCAGCATAACGAATTTAGCATTTATATTGGCAAAGATAGCAAATATAGCGCGCTGATTTTTGGCGGCGTGGATAAAACTTTTTTGAAGGCGATATTTATATGTTTCCGGTGGTGAAAGAA
TATTATTGGGAAATTCATTTTGATGGCCTGTATATTGATCATCAGAAATTTTGCTGCGGCGTGAACAGCATTGTGTATGATCTGAAAAAAAAAGATCAGGAAAACAACAAACTGTTTTTTACCCGCAAATATTTTCG
CAAAAACAAATTTAAAACCCATCTGCGCAAATATCTGCTGAAAAAATTAAACATCAGAAAAAACAGAAACATAGCAACCATAAAAAAAAAAAACTGAACAAAAAAAAAAAACTATCTGATTTTTGATAGCGGCACCA
GCTTTAACAGCGTGCCGAAAGATGAAATTGAATATTTTTTTCGCGTGGTGCCGAGCAAAAAATGCGATGATAGCAACATTGATCAGGTGGTGAGCAGCTATCCGAACCTGACCTATGTGATTAACAAAATGCCGT
TTACCCTGACCCCGAGCCAGTATCTGGTGCGCAAAAACGATATGTGCAAACCGGCGTTTATGGAAATTGAAGTGAGCAGCGAATATGGCCATGCGTATATTCTGGGCAACGCGACCTTTATGCGCTATTATTATA
CCGTGTATCGCCGCGGCAACAACAACAACAGCAGCTATGTGGGCATTGCGAAAGCGGTGCATACCGAAGAAAACGAAAAATATCTGAGCAGCCTGCATAACAAAATTAACAACCTGTAA

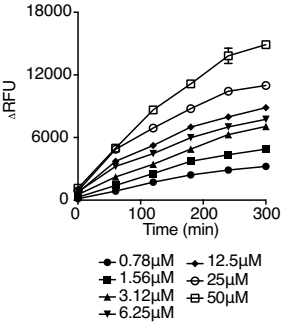
C



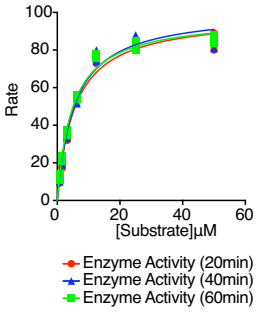
D



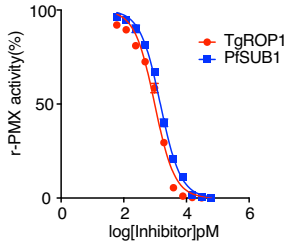
E



F



G



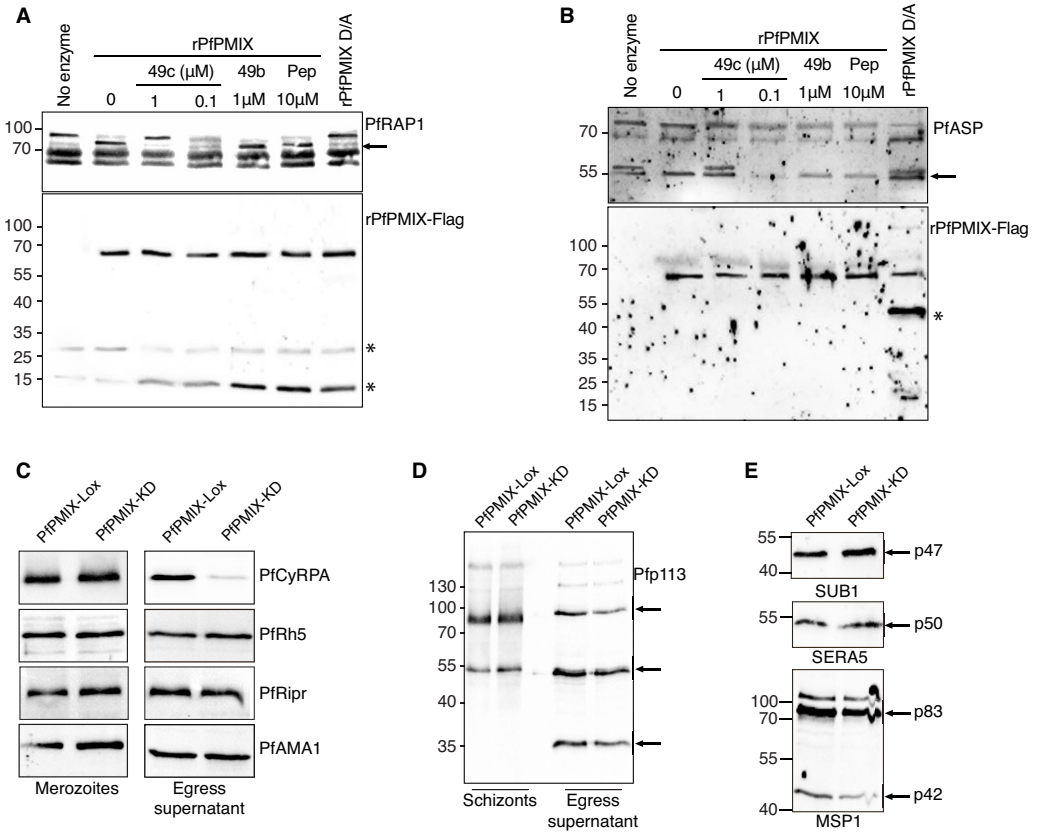
H

	TgROP1 peptide	PfSUB1 peptide
Vmax	97.64±2.6 RFU/min	N.D
Km	5.2±0.43 uM	N.D
IC50	0.9±0.2 nM	1.4±0.16 nM
Ki	0.19±0.02 nM	N.D

Figure S5:

A) Sequence of the codon remodeled *PfPMIX* gene used for heterologous recombinant protein expression. **B)** Sequence of the codon remodeled *PfPMX* gene used for heterologous recombinant protein expression. **C)** Cleavage assay showing cleavage of TgROP1 peptide by rPfPMIX which is inhibited in the presence of 1 μM 49c but not with 1 μM 49b or 10 μM pepstatin. rPfPMIX D/A is used as a control. **D)** Cleavage assay showing cleavage of TgROP1 peptide by rPfPMX which is completely inhibited in the presence of 10 nM 49c but not in the presence of 1μM 49b or 10μM pepstatin. 1nM 49c abrogates ~ 70% of cleavage. rPfPMIX D/A is used as control. **E)** Kinetics showing cleavage of the fluorogenic TgROP1 peptide at varying concentrations over time by rPfPMX. **F)** Michaelis-Menten curves showing the rate of cleavage (Rate=RFU/min) of increasing concentration of TgROP1 peptide by rPfPMX. The data were used to derive k_m and V_{max} values. **G)** Dose response curve showing inhibition of rPfPMX activity by 49c to determine IC_{50}. IC_{50} was determined against TgROP1 and PfSUB1 fluorogenic peptide. **H)** Table showing different kinetics parameter for rPfPMX.

Figure S6



G

	rPfPMIX	rPfPMX
PfRAP1 peptide	active	inactive
PfRAP1 ip	active	ND
PfASP from egress supernatant	active	ND
PfSUB1 peptide	inactive	active
rPvSUB1	ND	active
PfSUB1 peptide	inactive	active
PbCelTOS ip	ND	active
PfAMA1 peptide	inactive	active

H

	Outcome of selection		
Parasite strains	**3D7**	**W2**	**Dd2**
49c 10nM	negative	negative	negative
49c 100nM	negative	negative	negative
Chloroquine 100nM	9 weeks	ND	ND
Atovaquone 10nM	8 weeks	ND	ND
Pyrimethamine 10nM	12 weeks	ND	ND

Figure S6:

A-B) Immunoblot showing cleavage of **A)** PfRAP1 and **B)** PfASP in the presence of rPfPMIX. Cleavage was inhibited in the presence of 1μM 49c but not in the presence of 1 μM 49b and 10 μM pepstatin. No enzyme and rPfPMIX D/A are used as control. The blot is re probed with anti-Flag Ab to show the presence of rPfPMIX. **C)** 3D7-DiCre and PfPMIX-Lox parasites were treated with DMSO/rapamycin at ring stage and further synchronized with C2. When fully mature, merozoites were allowed to egress for 30 min, and free merozoites and egress supernatants were collected. Left: Immunoblot of the full protein extract from free merozoites and egress supernatant, using antibodies against PfCyRPA, PfRh5, PfRipr, and PfAMA1. **D)** Immunoblot evaluating the processing and the secretion of Pfp113 upon PfPMIX Knock-down. PfPMIX-Lox parasites were treated with rapamycin at ring stage or 49c (1 nM) for 10 h. When fully mature, schizonts were collected. **E)** Immunoblots evaluating the processing event of PfSUB1, PfSERA5 and PfMSP1 upon PfPMIX knock down. *P. falciparum* parasites were treated with rapamycin for 4 h at ring stage 4 h. When fully mature, schizonts were collected. Processed forms are indicated with arrows. **F)** Immunoblot showing cleavage of rPvSUB1 in the presence of rPfPMX. Cleavage is inhibited in the presence of 10 μM 49c but 1μM 49b and 10 μM pepstatin have no inhibitory affect. No enzyme and rPfPMX D/A are used as control in this experiment. **G)** Summary table of the different substrates tested in the cleavage assays. **H)** Summary table showing the results of the in vitro resistant parasites selections; No resistant parasites were obtained under 49c selection while resistant strains were readily obtained when selecting with chloroquine, atovaquone and pyrimethamine.

Figure S7

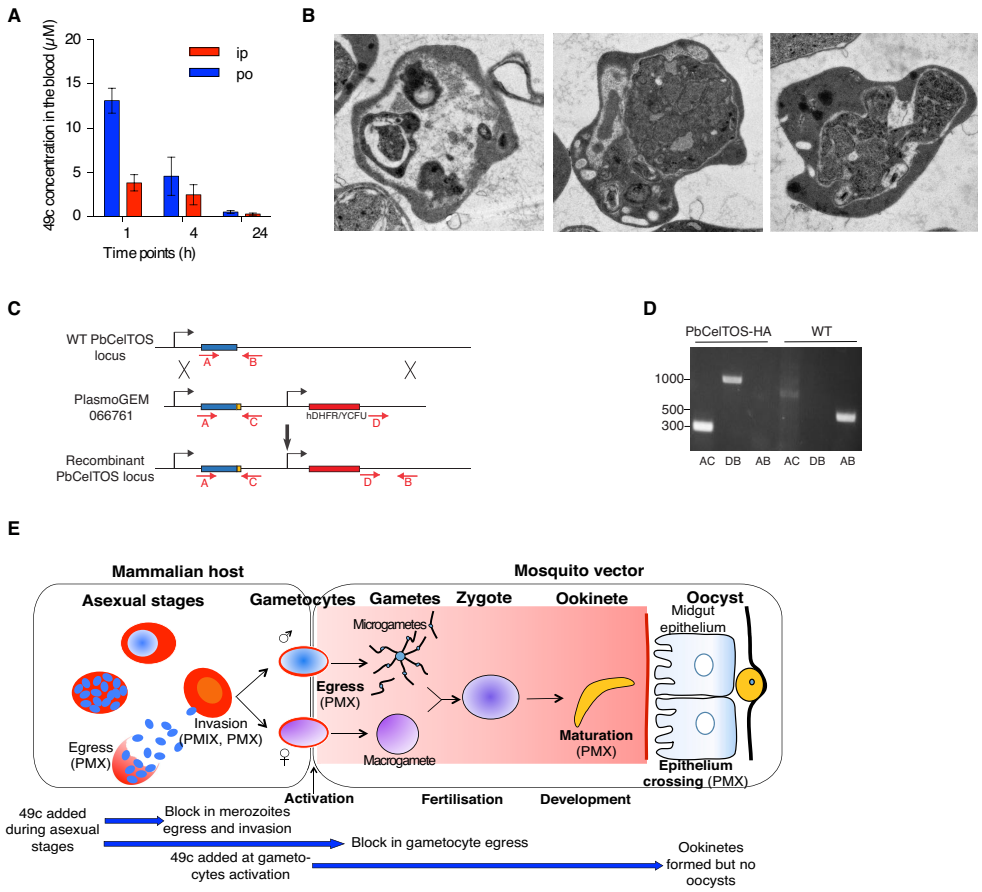


Figure S7:

A) Pharmacokinetics (PK) of the 49c compound in the mouse model. Concentrations of 49c were measured in sera 1, 4 and 24 h after 100 mg/kg *per os* administration or intra peritoneal (i.p.) injection. **B)** EM pictures of the circulating degenerating *P. berghei* schizonts observed in mice treated with 49c. **C)** Schematic of the strategy used to generate the PbCelTOS-HA strain. The primers used for genotyping are shown in red. **D)** Agarose gel of genotyping PCR reactions performed on genomic DNA extracted from a PbCeLTOS-HA parasite clone and parental line. **E)** Scheme showing the different stages of the malaria parasite, where PMIX and PMX are expressed and can be blocked by 49c.

Figure S8

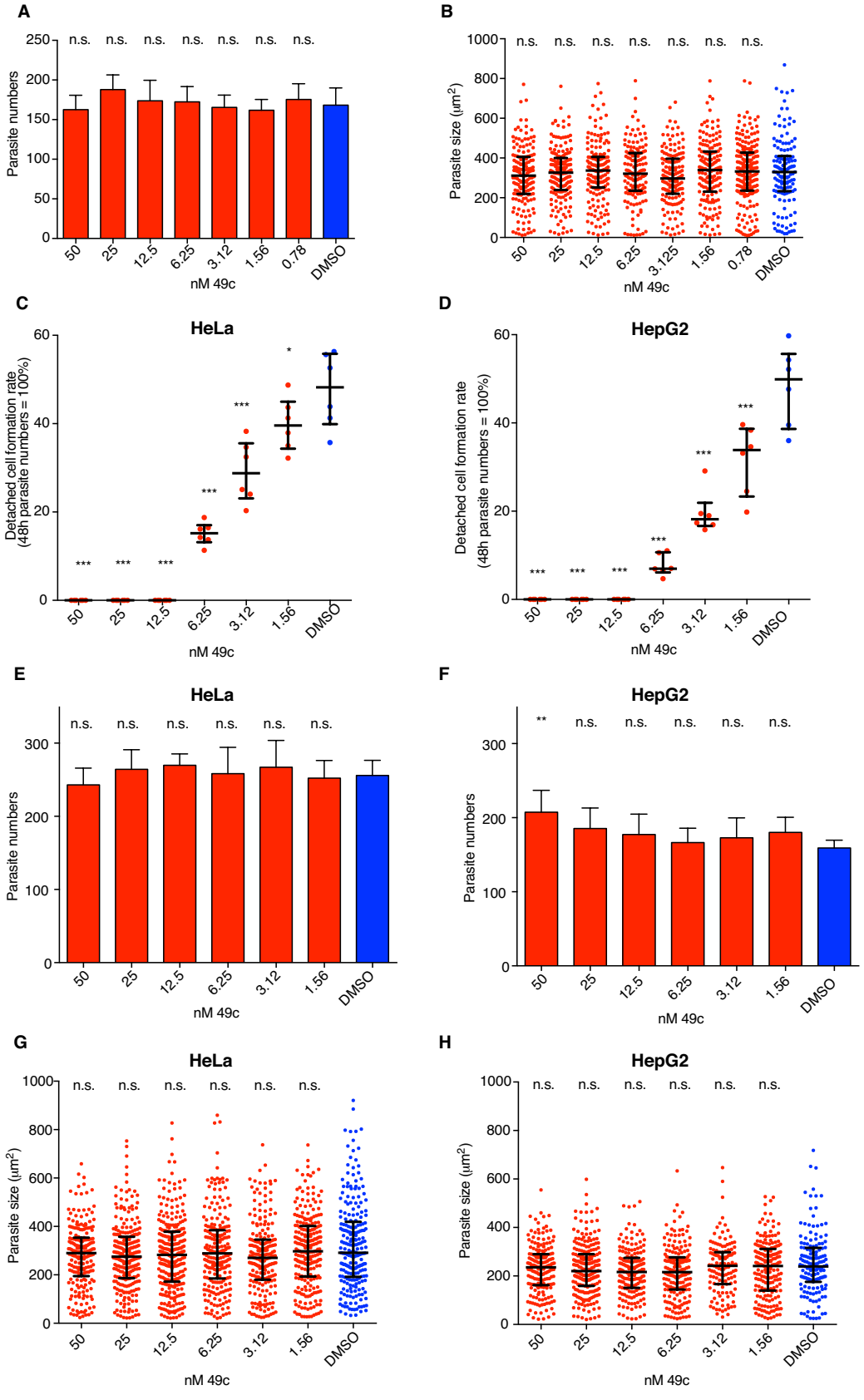

A
B
C
HeLa
D
HepG2
E
HeLa
F
HepG2
G
HeLa
H
HepG2
Parasite numbers
Parasite size (μm²)
Detached cell formation rate (48h parasite numbers = 100%)
nM 49c
50
25
12.5
6.25
3.12
3.125
1.56
0.78
DMSO
n.s.
*
**
***

Figure S8:

49c has no effect on parasite number and parasite size during liver stage development

A) Infected HeLa cells from Fig. 4A were analysed for parasite number (+/- SD, n=6) by automated microscopy (In Cell analyzer).B) Parasite sizes of one replicate of treated cells from A) are shown (median with interquartile range). C-H) Infection and treatments were done in parallel in HeLa and HepG2 cells. Detached cell formation rate (C and D), parasite number (E and F) and parasite sizes (G and H) are presented as in (Fig. 4A, Fig. S8 A and B).

One-way ANOVA with Dunnet's Multiple Comparisons (* $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$, ns/not significant > 0.05) showed no significant difference in size and numbers compared to the control treated cultures.

One-way ANOVA with Dunnet's Multiple Comparisons (* $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$, ns/not significant > 0.05) showed no significant difference in size and numbers compared to the control treated cultures.

Movie S1

Egress of PfPMIX-Lox *P. falciparum* **merozoites following release of a C2-mediated egress block.** Schizonts allowed to mature for 2 h in the presence of the PfPKG inhibitor C2 were washed, resuspended in fresh warm medium without C2 and immediately observed by time-lapse video microscopy.

Movie S2

Egress of PfPMIX-KD *P. falciparum* **merozoites, following release of a C2-mediated egress block.** Schizonts allowed to mature for 2 h in the presence of the PfPKG inhibitor

C2 were washed, resuspended in fresh warm medium without C2 and immediately observed by time-lapse video microscopy.

1. C. L. Ciana *et al.*, Novel in vivo active anti-malarials based on a hydroxy-ethyl-amine scaffold. *Bioorganic & medicinal chemistry letters* **23**, 658-662 (2013).
2. M. A. Child *et al.*, Regulated maturation of malaria merozoite surface protein-1 is essential for parasite growth. *Molecular microbiology* **78**, 187-202 (2010).
3. R. Stallmach *et al.*, Plasmodium falciparum SERA5 plays a non-enzymatic role in the malarial asexual blood-stage lifecycle. *Molecular microbiology* **96**, 368-387 (2015).
4. C. Crosnier *et al.*, A library of functional recombinant cell-surface and secreted P. falciparum merozoite proteins. *Mol Cell Proteomics* **12**, 3976-3986 (2013).
5. M. L. Tonkin *et al.*, Host cell invasion by apicomplexan parasites: insights from the co-structure of AMA1 with a RON2 peptide. *Science* **333**, 463-467 (2011).
6. K. S. Reddy *et al.*, Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for Plasmodium falciparum erythrocyte invasion. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 1179-1184 (2015).
7. P. Pino *et al.*, A tetracycline-repressible transactivator system to study essential genes in malaria parasites. *Cell host & microbe* **12**, 824-834 (2012).
8. F. Plattner *et al.*, Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell host & microbe* **3**, 77-87 (2008).

9. G. Rugarabamu *et al.*, Distinct contribution of Toxoplasma gondii rhomboid proteases 4 and 5 to micronemal protein protease 1 activity during invasion. *Molecular microbiology* **97**, 244-262 (2015).

10. T. Chu *et al.*, Genetic evidence strongly support an essential role for PfPV1 in intra-erythrocytic growth of P. falciparum. *PloS one* **6**, e18396 (2011).

11. C. Pfander *et al.*, A scalable pipeline for highly effective genetic modification of a malaria parasite. *Nat Methods* **8**, 1078-1082 (2011).

12. C. R. Collins *et al.*, Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. *Molecular microbiology* **88**, 687-701 (2013).

13. C. Lambros *et al.*, Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol* **65**, 418-420 (1979).

14. P. K. Harris *et al.*, Molecular identification of a malaria merozoite surface sheddase. *PLoS Pathog* **1**, 241-251 (2005).

15. J. A. Thomas *et al.*, Development and Application of a Simple Plaque Assay for the Human Malaria Parasite Plasmodium falciparum. *PloS one* **11**, e0157873 (2016).

16. C. R. Collins *et al.*, Malaria parasite cGMP-dependent protein kinase regulates blood stage merozoite secretory organelle discharge and egress. *PLoS Pathog* **9**, e1003344 (2013).

17. C. Withers-Martinez *et al.*, Plasmodium subtilisin-like protease 1 (SUB1): insights into the active-site structure, specificity and function of a pan-malaria drug target. *International journal for parasitology* **42**, 597-612 (2012).

18. M. J. Blackman *et al.*, Structural and biochemical characterization of a fluorogenic rhodamine-labeled malarial protease substrate. *Biochemistry* **41**, 12244-12252 (2002).

19. C. Withers-Martinez *et al.*, The malaria parasite egress protease SUB1 is a calcium-dependent redox switch subtilisin. *Nat Commun* **5**, 3726 (2014).

20. D. J. Fitzgerald *et al.*, Protein complex expression by using multigene baculoviral vectors. *Nat Methods* **3**, 1021-1032 (2006).

21. M. Buchs *et al.*, High-throughput insect cell protein expression applications. *Methods in molecular biology* **498**, 199-227 (2009).

22. J. A. Boddey *et al.*, An aspartyl protease directs malaria effector proteins to the host cell. *Nature* **463**, 627-631 (2010).

23. M. J. Coffey *et al.*, An aspartyl protease defines a novel pathway for export of Toxoplasma proteins into the host cell. *Elife* **4**, (2015).

24. D. W. Wilson *et al.*, Macrolides rapidly inhibit red blood cell invasion by the human malaria parasite, Plasmodium falciparum. *BMC Biol* **13**, 52 (2015).

25. D. T. Riglar *et al.*, Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. *Cell host & microbe* **9**, 9-20 (2011).

26. L. Chen *et al.*, An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by Plasmodium falciparum. *PLoS Pathog* **7**, e1002199 (2011).

27. G. R. Mair *et al.*, Universal features of post-transcriptional gene regulation are critical for Plasmodium zygote development. *PLoS Pathog* **6**, e1000767 (2010).

28. M. Brochet *et al.*, Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca(2)(+) signals at key decision points in the life cycle of malaria parasites. *PLoS biology* **12**, e1001806 (2014).

29. P. C. Burda *et al.*, A Plasmodium phospholipase is involved in disruption of the liver stage parasitophorous vacuole membrane. *PLoS Pathog* **11**, e1004760 (2015).

30. M. De Niz *et al.*, The machinery underlying malaria parasite virulence is conserved between rodent and human malaria parasites. *Nat Commun* **7**, 11659 (2016).