A comprehensive model for assessment of liver stage therapies targeting *Plasmodium vivax* and *Plasmodium falciparum*

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Malaria liver stages represent an ideal therapeutic target with a bottleneck in parasite load and reduced clinical symptoms; however, current in vitro pre-erythrocytic (PE) models for *Plasmodium vivax* and *P. falciparum* lack the efficiency necessary for rapid identification and effective evaluation of new vaccines and drugs, especially targeting late liver-stage development and hypnozoites. Herein we report the development of a 384-well plate culture system using commercially available materials, including cryopreserved primary human hepatocytes. Hepatocyte physiology is maintained for at least 30 days and supports development of *P. vivax* hypnozoites and complete maturation of *P. vivax* and *P. falciparum* schizonts. Our multimodal analysis in antimalarial therapeutic research identifies important PE inhibition mechanisms: immune antibodies against sporozoite surface proteins functionally inhibit liver stage development and ion homeostasis is essential for schizont and hypnozoite viability. This model can be implemented in laboratories in disease-endemic areas to accelerate vaccine and drug discovery research.
Malaria is a major global disease with almost half of the world’s population at risk, resulting in an estimated 216 million cases and 445,000 deaths in 2015. The two most prevalent causes of malaria are apicomplexan parasites *Plasmodium falciparum*, the most virulent and dominant species in Sub-Saharan Africa, and *P. vivax*, with the widest geographic distribution and major economic impact. *Plasmodium* sporozoites initiate infections when injected into the dermis by a female anopheline mosquito and then use a molecular motor-driven mechanism to rapidly enter the human circulatory system and translocate across the liver sinusoid. After invasion of hepatocytes, liver-resident parasites undergo asexual schizogony to form tens of thousands of merozoites. Following merozoite egress from the infected liver cell, the parasites escape into the hepatic vein to infect erythrocytes where they asexually replicate in circulation, leading to geometric population expansion and the clinical symptoms of malaria. Although it is the blood-stage infection that causes clinical disease, the sporozoite and the liver stage (LS) forms, which together are referred to as pre-erythrocytic (PE) stages, represent a vulnerable bottleneck in infection that causes clinical disease, the sporozoite and the infected liver cell, the parasites escape into the hepatic vein to infect erythrocytes where they asexually replicate in circulation, leading to geometric population expansion and the clinical symptoms of malaria. Although it is the blood-stage infection that causes clinical disease, the sporozoite and the liver stage (LS) forms, which together are referred to as pre-erythrocytic (PE) stages, represent a vulnerable bottleneck in infection that causes clinical disease, the sporozoite and the infected liver cell, the parasites escape into the hepatic vein to infect erythrocytes where they asexually replicate in circulation, leading to geometric population expansion and the clinical symptoms of malaria. Therefore, chemotherapeutic and immunoprophylactic interventions have converged on targeting *P. vivax* and *P. falciparum* PE stages as a strategy to block progression to clinical malaria and transmission.

The biology of the *P. vivax* and *P. falciparum* LS forms fundamentally differ as some *P. vivax* parasites will remain quiescent as uninucleate stages termed hypnozoites. Hypnozoites are not susceptible to the mechanism of action of most antimalarial drugs and can persist for weeks, months, or even years before an unknown re-activation mechanism stimulates completion of development and a symptomatic blood-stage infection. In addition, *P. vivax* is able to rapidly form transmissible gametocytes in circulation before presentation of clinical symptoms. Therefore, an effective malaria elimination toolbox has been proposed consisting of a multi-stage drug with hypnozocticidal activity and a highly efficacious vaccine conferring life-long sterile immunity; however, neither of these tools is currently available. Malaria control is now focused on treating the symptomatic blood and transmission stages with front-line drugs of Artemisinin Combination Therapies (ACTs) for *falciparum* malaria and predominately chloroquine (CQ) for *vivax* malaria. Prophylactic regimens of atovaquone and proguanil (Malarone) target only the LS schizont, while the only chemotherapeutic intervention currently capable of targeting hypnozoites are 8-aminoquinolines, such as primaquine and tafenoquine. Unfortunately, use of 8-aminoquinolines is contraindicated in many malaria endemic countries because of its toxicity in individuals with some glucose-6-phosphate dehydrogenase (G6PD) polymorphisms, restricting mass drug administration campaigns in regions where high-risk favisms are common. In regards to malaria vaccination, development of PE vaccines has focused on the initial stages of infection targeting antibodies to the sporozoite surface to neutralize parasite migration to the liver and consequently the disease-causing blood stage. However, vaccines to prevent malaria have lagged far behind drug development efforts as only one vaccine for *P. falciparum* has been licensed, RTS,S/SAS01 or Mosquirix TM. In Phase III clinical field trials Mosquirix TM showed a temporary, age-specific response with only partial protection. Meanwhile, vaccines for *P. vivax* remain mostly in the pre-clinical discovery phase of development and only a few candidates have progressed into initial clinical trials.

In vitro PE assays are essential for preclinical assessment of novel vaccines and drugs, yet currently available PE assays are inadequate for meeting the demands of a genuine PE screening effort. Historically, many studies of *Plasmodium* liver models used human hepatoma lines, which are deficient in specific surface receptors present on primary human hepatocytes (PHHs) that are required for *Plasmodium* sporozoite invasion, resulting in poor infection rates. Furthermore, LS formation within hepatoma cells is atypical compared to that noted in animal models as the schizonts are smaller and cannot be as easily distinguished from hypnozoites and persistent proliferation of these host cells hinders image-based analysis. More recent studies have used fresh, cryopreserved PHHs, or human iPSC-derived hepatocyte-like cells in a 96-well plate co-culture model, yet LS development rates (LS parasites per sporozoite inoculum) remained low despite sporozoite infection loads 10-fold higher than what we report herein. Animal models engrafted with PHHs offer excellent *Plasmodium* LS development but intrinsically high costs and low-throughput hinders the use of this model for drug discovery.

In this report we describe a robust anti-PE therapeutic screen streamlined for *P. vivax* and *P. falciparum* using a PHH culture system comprised entirely of commercially available 384-well plates and cell culture reagents. Reducing to a 384-well microtiter format promotes key morphological and functional characteristics of native in situ hepatocytes and allows for high-resolution imaging, seamless image acquisition with faster imaging speed, and integration of automated high-content image analysis. Identification of optimal sporozoite isolation and hepatocyte culture methods resulted in highly reproducible formation of hundreds of LS parasites in each well of a 384-well plate, making the model ideal for *P. vivax* and *P. falciparum* PE bioassays. Implementation of our PHH culture system in laboratories located in and outside of malaria endemic areas demonstrates this novel PE model will help fill a critical technology gap hindering advancement of PE-active therapies and lay the foundation for next-generation malaria control and elimination.

**Results**

**Functional human hepatocytes in a commercial 384-well plate.** We discovered that the small-scale collagen-treated surface area of particular commercially available 384-well plates coupled with our methodology provides a suitable microphysiological environment for long-term cultivation of PHHs (Fig. 1a, Supplementary Fig. 1a). The PHH donor lots PDC and NLX, used for the majority of studies described herein, were seeded at a density of 1.8 × 10⁴ live cells per well to achieve attachment of ~1 × 10⁴ cells per well. High-content imaging (HCl) of the cultured PHHs revealed individual cells in a confluent layer organized into lobule-like multicellular units. A rapid re-acquisition of primary cell characteristics occurred within two days, including visible transport of biliary metabolites and active mitochondria, and then remained stable for at least 30 days (Fig. 2a).

Functionality and metabolic activity of cultured PHHs were further characterized by several biometrics. Albumin production leveled at day 4 and remained stable thereafter with slight variation between 2 and 4 mg ml⁻¹ for a duration of 30 days (Fig. 2b, Supplementary Fig. 1b). Furthermore, PHHs maintained a CYP3A4 response to Rifampicin between days 4 and 20 along with stable factor IX expression for 3 weeks (Supplementary Fig. 1c, d). Hepatobiliary transport (active and inactive) of the bile canaliculi was measured by staining with CellTracker TM and quantified by image analysis. This biomarker passes into hepatocytes whereupon it is trapped as cell-impermeable glutathione methylfluorescein (GS-MF) that is subsequently actively transported into the bile canaliculi. Accumulation of this biliary metabolite can be used to quantify the functional
Radical cure drug response assays are ended at day 8 to allow for treatment and clearance of susceptible liver forms of *P. falciparum* on day 2. (BIVT) supported higher experimental matrix to include a range of seeding densities and PHH donor lots from four companies and subjected each to an experimental study of the major human malaria parasites, in vitro liver platform were expected to be highly supportive for in vivo development of *P. vivax* and *P. falciparum* LS parasite development rates than lots tested from other sources. We therefore decided to screen an additional 13 BIVT PHH donor lots for both *P. vivax* and *P. falciparum* development relative to lot PDC and the terminally-differentiated HepaRG hepatocyte line (as a negative control for LS development). Four donors, lots H, I, P, and Q, were significantly higher in LS development rate relative to PDC (*P < 0.0001 and *P < 0.05*, two-way ANOVA followed by Dunnnett’s multiple comparisons) while two donors, lot F and N did not support *P. falciparum* or *P. vivax* LS parasite growth. We also discovered some donors, lots G, H, I, and P, appeared susceptible to only *P. falciparum* or *P. vivax* LS parasite growth. The PHH donors that did not form a monolayer in the 384-well microplate.

Following characterization of *Plasmodium* spp. sporozoite infection rate, this expanded set of PHH donor lots were assessed for basal metabolic CYP activity (Supplementary Table 2, Supplementary Fig. 5) and hepatic functionality by measurement of bile canaliculi formation and mitochondrial activity over 14 days (Supplementary Table 3). Interestingly when measured over days 2–8, PHH donors found to have a stable mean TMRM intensity (≥3000 MFI), increase of cytoplasmic-stained cell area (≥30%), and a decrease of total hepatocytes appeared to support higher *Plasmodium* LS developmental rates. Furthermore, PHH donor lots with only a 2-fold increase in mean TMRM intensity and increased amount of inactive transport on day 8 (≥50%) had attenuated *Plasmodium* LS development with a reduction in mean LS schizont area. Lastly, the absence of LS parasite development was only observed in PHH donor lots with a decrease in active transport and minimal change in mean TMRM intensity from day 2 to 8 (Supplementary Table 3). After identification of suitable PHH donor lots, other microenvironmental factors that might influence sporozoite invasion and LS development were optimized. Hepatocyte seeding density and time between seed

**Complete development of *P. vivax* and *P. falciparum* LS parasites.** To evaluate our model’s suitability for experimental studies with human malaria parasites, we tested cryopreserved PHH donor lots from four companies and subjected each to an experimental matrix to include a range of seeding densities and sporozoite isolation methods. In our hands, three lots tested from Bioreclamation IVT (BIVT) supported higher *P. vivax* and *P. falciparum* LS parasite development rates than lots tested from other sources (Supplementary Fig. 4a, Supplementary Table 1). We therefore decided to screen an additional 13 BIVT PHH donor lots for both *P. vivax* and *P. falciparum* development relative to lot PDC and the terminally-differentiated HepaRG hepatocyte line (as a negative control for LS development). Four donors, lots H, I, P, and Q, were significantly higher in LS development rate relative to PDC (*P < 0.0001 and *P < 0.05*, two-way ANOVA followed by Dunnnett’s multiple comparisons) while two donors, lot F and N did not support *P. falciparum* or *P. vivax* LS parasite growth. We also discovered some donors, lots G, H, I, and P, appeared susceptible to only *P. falciparum* or *P. vivax* (Fig. 3a). Three PHH donors did not form a monolayer in the 384-well microplate.

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and sporozoite infection were identified as crucial variables; the highest \textit{P. vivax} LS development rates resulted from a seeding density of 1.8 $\times$ 10$^4$ live PHHs yielding a monolayer of ~1.0 $\times$ 10$^4$ cells, followed by a delayed sporozoite infection until at least day 3 to allow for monolayer assembly (Supplementary Fig. 4b–d).

The \textit{P. vivax} sporozoites produced from mosquitoes fed on clinically-isolated blood meals consistently yielded superior LS developmental rates (2–8.30%) compared to sporozoites from mosquito infections derived from in vitro-cultured \textit{P. falciparum} NF54-WT (0.60–2%) and \textit{P. falciparum} NF54-GFP (0.04–0.40%) following an inoculum of 5.0 $\times$ 10$^3$ sporozoites (Fig. 3b, f, g). Characterization of day 6 LS parasites revealed synchronized growth of \textit{P. falciparum} with 75.95% of the parasites having a mean parasite area of 73.82 ± 6.52 µm$^2$ ($n = 3$, $n' = 8$) (Fig. 3c, f).

Inversely, \textit{P. vivax} day 6 parasites developed asynchronously as the schizont population had larger, irregular parasite area ranging from 31.65 ± 0.97 to 1270 ± 197.30 µm$^2$ ($n = 3$, $n' = 26$). By day 8, some \textit{P. falciparum} LS schizonts successfully completed maturation and released infectious merozoites leading to further variability in LS...
Furthermore, *P. vivax* LS schizonts continued to grow remarkably larger from day 6 and nearly doubled in area with 32.44% LS having a mean area of 2709 ± 333.20 µm² (*n* = 3, *n*’ = 26) (Fig. 3d, g and Supplementary Table 4). Importantly, from days 6 to 8, *P. vivax* LS hypnozoites represented ~60% of the total LS parasites per well with a mean size of 34.55 ± 2.37 µm² (*n* = 3, *n*’ = 26) (Fig. 3e).

Developing LS forms of *P. vivax* and *P. falciparum* were analyzed by HCI using a panel of *Plasmodium* antibodies.
targeting proteins expressed at different stages of maturity (Fig. 4a–c). The *P. vivax* hypnozoites and schizonts were distinguishable by size beginning on day 4 post-infection with distinct individual merozoites evident in mature day 8 schizonts. In addition to the small sizes, hypnozoites stained positively for Upregulated In Infectious Sporozoite-4 (UIS4), a parasitophorous vacuole membrane (PVM) protein as previously noted, but were negative for both PVM markers Exported Protein-1 (EXP-1) and Exported Protein-2 (EXP-2) which function in active export in developing LS schizonts (Fig. 4a)\(^3\),\(^3\),\(^9\). Notably, day 8 *P. vivax* LS schizonts showed an accumulation of EXP-2 protein in unknown vacuoles suggesting a role in degradation or sequestration (Fig. 4b). All multinucleated developing stages of *P. vivax* and *P. falciparum* were stained positively for Acp, Acyl Carrier Protein (ACP), providing evidence of active fatty acid biosynthesis and glycolysis\(^3\). In addition, mature LS schizonts had high cytoplasmic expression of Macrophage Migration Inhibitory Factor (MIF) and Heat Shock Protein 70 (HSP70) confirming these parasites expressed proteins to modulate the human host (Fig. 4b, c)\(^3\). Mature hypnozoites stained negative for Merozoite Surface Protein 1 (MSP1) whereas day 8 LS schizonts showed MSP1 surface localization on segmenting merozoites (Fig. 4a). Day 5 *P. falciparum* LS schizonts were positive for both MSP1 and MSP2, even though the diffuse cytoplasmic localization indicated merozoite segmentation was in process (Fig. 4c)\(^4\),\(^5\). Interestingly, a portion of *P. vivax* late-stage schizonts stained positive for Pvs16, a gametocyte-specific protein, suggesting some LS merozoites are pre-committed as first generation gametocytes prior to the erythrocytic cycle (Fig. 4d and Supplementary Fig. 7). Finally, to confirm complete LS parasite maturation in the 384-well PHH culture system, merozoite infectivity was evaluated by addition or overlays of highly enriched human CD 71\(^+\) reticulocytes to *P. vivax* LS cultures (days 9–11) and fresh human infected erythrocytes. This established a 12 h and continued culture progressed to an asynchronous population at > 1% parasitemia. White scale bars represent 5 \(\mu\)m, gray scale bars represent 10 \(\mu\)m.

Fig. 4 High-resolution immunofluorescent identification of *Plasmodium* liver stage (LS) parasites. a High-resolution images of *P. vivax* hypnozoites demonstrate these forms have minimal nuclear material and are negative for schizogony markers EXP1, EXP 2 and MSP1. Hypnozoites stain positive for cytosolic markers MIF, HSP70, and GAPDH and reveal a functioning apicoplast. b By day 8, *P. vivax* schizonts show four times larger than the host cell hepatic nucleus, feature genome replication and segmentation, and stain positive for EXP1, EXP2, ACP, and MSP1. c The LS of *P. falciparum* is shorter than that of *P. vivax* schizonts; developing parasites are correspondingly less small. By day 5 merozoite segmentation has begun (as noted by ACP staining of separate apicoplasts) but not complete (as noted by diffuse staining of MSPs). d Immunofluorescent staining of day 8 *P. vivax* LS schizonts with anti-Pvs16, a sexual stage-specific biomarker for immature gametocytes, showed co-localization with developing LS merozoites indicated by segmented DNA. However, anti-Pvs16 signal does not appear in every LS. e The PHH system successfully supports complete maturation of *Plasmodium* LS schizonts measured by breakthrough into blood stage using reticulocyte (*P. vivax*, days 9–11) or RBC (*P. falciparum*, days 7–8) overlays with initial giemsa staining every 6 h. *P. vivax* overlays show formation of merozoite packages which rupture into the reticulocyte culture leading to invasion. Alternatively, no merozoites were captured in *P. falciparum* overlays but early rings were present within the first 12 h and continued culture progressed to an asynchronous population at > 1% parasitemia.
red blood cells (RBCs) to *P. falciparum* cultures (days 7–8). Within first 24 h of *P. vivax* overlays, merozoite packages (merosomes) were seen released into the collected media along with *P. vivax*-infected reticulocytes and although we did not attempt to observe *P. falciparum* merosomes, early ring-infected RBCs were detected and continued development within RBCs was observed (Fig. 4e).

**Design consideration for treatment mode and HCI endpoint.** Ideally, a *P. vivax* and *P. falciparum* PE therapeutic screening platform should capture anti-parasite inhibitory activity against sporozoite migration, hepatocyte infection, LS development, and blood-stage breakthrough. To achieve these objectives, assay designs were considered either prophylactic, to prevent infections, or radical cure, to eliminate existing infections (Fig. 1b). For evaluating prophylactic efficacy of vaccines, sporozoites were exposed to immune antibodies or sera at the time of or just prior to their addition to the hepatocyte cultures. Similarly, prophylactic drugs were evaluated for activity by exposure at the time of sporozoite infection. However, LS assays for *P. vivax* require evaluation for radical cure activity to eliminate mature hypnozoites, as early drug application (prior to day 4–5 post-infection) can be effective on immature hypnozoites but inactive on mature hypnozoites (day 5 and beyond)24. Regardless of treatment mode, all cultures were fixed prior to release of merozoites to enable HCI-based quantification of growth using our experimentally defined LS phenotypes based on size and differential protein expression (UIS4 and GAPDH) (Table 1). In our 4-step HCI-based screening assays, antibody reagents were chosen for their staining patterns to facilitate automated quantification by defining the full LS form to enable measurement of growth area by automated morphometric analyses. Quantified populations were then normalized to controls and dose response curves fitted to generate half-maximal effective concentration (EC_{50}) and inhibitory concentration (IC_{50}) in Collaborative Drug Discovery (CDD) Vault or Graphpad Prism (Fig. 5). Automated imaging and quantification on an Operetta or ImageXpress was highly robust with 95–100% accuracy in identifying developing LS schizonts and hypnozoites when compared to conventional manual techniques (Table 2). Overall, automated HCI rapidly increased screening throughput allowing at least three 384-well plates to be analyzed within 24 h on an Operetta using wide field mode and 8 h on an ImageXpress spinning disc confocal imaging system.

**Anti-CSP antibodies show species-specific inhibition.** Next, our LS platform was validated for inhibition of liver stage developmental assays (ILSDA; Fig. 1b) by examining specificity and sensitivity of well-characterized monoclonal antibodies (mAbs), anti-PfCSP mAB 2A10, anti-PvCSP mAB 2F2, and PvCSP mAB 2E10.E913–47. Several ILSDA protocol optimizations lead to improved experimental results. First, we discovered that sporozoites of both species exposed to mAbs in phosphate buffered saline (PBS) adversely affected ILSDA outcomes by significantly decreasing sporozoite viability, hepatocyte infection rates, and increasing false positives; therefore, a buffered cell culture medium (RPMI or hepatocyte culture media) was used for incubation with mAB (Supplementary Fig. 8a, b)24,45,48–50. Second, having found sporozoite invasion rates were improved by allowing the sporozoite–hepatocyte interaction to persist for 24 h instead of the conventional 3-h interaction followed by a wash step, ILSDAs were performed by co-incubation of sporozoites, mAb, hepatocytes, and media overnight instead of the typical sporozoite pre-treatment with mAB. Third, anti-PvCSP mAB 2F2 exhibited a minimal difference in IC_{50} values for sporozoites exposed to the mAB for 20 min prior to addition to hepatocytes vs. co-incubation of mAb, sporozoites, and hepatocytes (Supplementary Fig. 8c). Finally, the assay endpoint was extended to day 6 or 8 in order to identify potential late-stage developmental phenotypes that are not easily evaluated with current LS platforms using hepatoma cells.

Our HCI analysis at 6 days post-infection confirmed that anti-PvCSP mAB 2F2 completely inhibited *P. vivax* sporozoite invasion and LS development at concentrations of 250 µg ml^{-1} with an IC_{50} of 4.60 µg ml^{-1} (n = 3, n’ = 2) (Fig. 6a). This inhibition was specific, as *P. falciparum* sporozoites exposed to anti-PvCSP mAB 2F2 resulted in normal *P. falciparum* LS development and *P. vivax* sporozoites exposed to anti-PfCSP mAB 2A10 resulted in normal *P. vivax* LS development. These results were expected as anti-P. falciparum and *P. vivax* sporozoite immunity (natural immunity or by immunization with irradiated sporozoites) is directed towards the dominant Bc epitopes of the central repeat regions of mammalian CSPs, which are the epitope targets of these anti-CSP mAbs. Similar analysis of *P. vivax* sporozoite attenuation with anti-PvCSP mAB 2F2 revealed a novel discovery that *P. vivax* LS parasites were significantly reduced in size and had lower DNA content, indicating post hepatocyte-invasion antibody inhibition of LS development (Fig. 6b, c). Moreover, additional day 8 ILSDAs performed with cryopreserved *P. vivax* VK247-positive sporozoites and anti-2E10.E9 mAB (targeting CSP subtype VK5247) obtained a similar IC_{50} (2.85 µg ml^{-1}) compared to ILSDAs with fresh *P. vivax* sporozoites, indicating a possible use of cryopreserved sporozoites for standardized screening (Supplementary Fig. 9)15,32. While further study is needed, these results show our platform is suitable for gaining a deeper understanding of the immune response to *P. vivax* sporozoites.

### Table 1 High-content imaging and analysis of *Plasmodium* liver stage (LS) parasites

| Parasite population | Size category | Area          | Mean intensity^a | Maximum intensity^a | Cell roundness |
|---------------------|--------------|---------------|------------------|---------------------|---------------|
| Small forms:        |              |               |                  |                     |               |
| 1                   | >15 µm^2<100 µm^2 | >1500          | >3000            | >0.80               |               |
| Large forms:        |              |               |                  |                     |               |
| A                   | >99 µm^2<300 µm^2 | >800           | >1500            | >0.70               |               |
| B                   | >299 µm^2<500 µm^2 | >250           | >1000            | >0.70               |               |
| C                   | >499 µm^2<1000 µm^2 | >250           | >1000            | >0.70               |               |
| D                   | >999 µm^2<2500 µm^2 | >200           | >300             | >0.60               |               |
| E                   | >2499 µm^2 | >200           | >600             | >0.38               |               |

^a Based on FITC channel

Defined fluorescent imaging parameters and thresholds used to identify and quantify *Plasmodium* LS parasites using Perkin Elmer Harmony software. Small forms refer to *P. falciparum* hypnozoites (mean ~30 µm^2) but with wide size range also represents for *P. falciparum* day 6 schizonts (mean ~80 µm^2). Large forms refer to developing schizonts and are further categorized into 5 distinct groups based upon area and intensity.
understanding of the activity of anti-CSP antibodies as we identified and quantified inhibitory effects manifesting during and after hepatocyte invasion.

Similar species-specific concentration-dependent results were obtained for *P. falciparum* sporozoite infection and development in PHHs using anti-PICSP mAB 2A10. Conversely, anti-PvCSP mAB 2F2 had no measurable effect on *P. falciparum* development at any concentration (Fig. 6d). The higher concentrations of anti-PICSP mAB 2A10 of 80 and 40 µg ml⁻¹ led to a 100% functional inhibition of parasite development with elevated reduction (>50% inhibition) in parasite development across all remaining concentrations, respectively (Fig. 6d). Similar to the *P. vivax* ILSDA, *P. falciparum* sporozoites exposed to lower concentrations of antibody (10 µg ml⁻¹) permitted hepatocyte infection but resulted in late-stage LS developmental growth defects and delayed maturation (Fig. 6e, f). This consistently observed growth attenuation activity suggests that in addition to invasion inhibition, an anti-sporozoite antibody can attenuate intracellular LS development through an unknown mechanism.

To determine the utility of the ILSDA to experimentally evaluate functional immunogenicity of novel vaccines, *P. vivax* and *P. falciparum* sporozoites were treated with mouse immune sera samples (G2, G3, G6, and G7) raised against a self-assembling protein nanoparticle (SAPN) (FMP014/ALF, FMP014/ALFQ, FMP014V/ALF, and FMP014V/ALFQ, respectively) [3]. Samples G2 and G3 were derived from immunization with the FMP014 monomer containing PICSP CD4⁺ and CD8⁺ epitopes, universal T₁ epitopes, portions of the α-TSR domain, and 6 repeats of the NAMP motifs of the PICSP with differing Army Liposomal Formulation (ALF) based adjuvants (ALF or ALQ) [3]. Samples G6 and G7 were derived by immunization with an SAPN assembled from an FMP014V monomer containing two copies of the PvCSP (VKS type 210) repeat region motif and the α-TSR domain of PICSP [3]. Invasion and development of *P. falciparum* sporozoites were inhibited in a dose-dependent manner similar to the anti-CSP mAB (Fig. 6g) for all four serum samples with G2 exhibiting the strongest inhibitions for all dilutions and a growth phenotype at 1:16 dilution. Alternatively,

### Table 2 Comparison of *P. vivax* liver stage (LS) parasite quantification: Operetta vs. Manual

| Harmony software | Manual accuracy | Accuracy |
|------------------|----------------|----------|
| **Small forms** | **Large forms** | **Small count** | **Large count** |
| well 1 180 | 266 | 179 | 252 | 99.40% | 99.40% |
| well 2 182 | 249 | 174 | 243 | 95.40% | 97.50% |
| well 3 180 | 256 | 176 | 256 | 97.70% | 100% |

Perkin Elmer Harmony software exhibits high level precision of identifying *P. vivax* LS parasites with >95% accuracy.

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**Fig. 5** Enhanced-throughput imaging and analysis for PE therapeutic screening. Schematic represents automated imaging and data analysis workflow using the Operetta high-content imaging (HCI) system (Perkin Elmer) where four consecutive steps are followed for maximum throughput of liver stage (LS) screening. Step 1: fluorescent channels are selected based on secondary conjugates and imaging parameters are set based on ×20 magnification (0.4 NA). Step 2: LS parasites are identified by high intensity staining patterns of LS-specific antibody (i.e., anti-rUIS4 for *P. vivax* or anti-GAPDH for *P. falciparum*) denoted by line plot profile (1, 2). Step 3: LS parasites are further characterized into size categories by area and roundness to ensure software is correctly selecting the entire parasite. Step 4: After LS parasites are quantified, raw data is imported into CDD vault or Graphpad Prism where % inhibition and dose-response curves are calculated based on total parasites and size. *P. vivax* LS assays specifically determine compound activity on hypnozoite population. White scale bars represent 5 µm.
sample G6 only moderately inhibited *P. vivax* sporozoites and G7 showed 100% inhibition at lowest dilutions with an IC⁵₀ at 1:8 dilution (*n* = 2, *n' = 2) (Fig. 6g). The pre-bleed serum control showed minimal inhibitory effect compared to the unexposed sporozoite control (Supplementary Fig. 8d). To overcome experimental bias, all percent inhibition calculations were standardized to the pre-bleed serum control⁴⁸.

**Compound library screen targeting LS parasites.** Several compound collections were screened for activity against *P. vivax* LS parasites in our LS platform following the drug treatment modes described in Fig. 1. Compounds from the MMV portfolio were tested in eight-point dose response assays, including triplicate controls (KDU691 and atovaquone) in addition to 36 compounds per plate, with concentrations ranging from 10 µM to 5 nM represented in singleton wells. In every assay plate, potent activity of phosphatidylinositol 4-kinase inhibitor (PI4K) KDU691 was confirmed against all forms except hypnozoites treated in the radical cure mode and atovaquone activity only against schizonts treated in prophylactic mode (Fig. 7a). To demonstrate multispecies capability and cross-species comparisons, a small subset of 6 MMV compounds were evaluated in the eight-point dose response assay against *P. falciparum* LS schizonts (Fig. 7b and Supplementary Table 1). Compounds were tested in duplicate in two independent experiments in prophylactic mode only, as *P. falciparum* does not produce hypnozoites and completes development prior to the radical cure treatment times. Comparison of EC⁵₀ values between *P. vivax* and *P. falciparum* revealed similar efficacies between atovaquone, DSM421, P218, and KAF156. Pyrimethamine showed a potent EC⁵₀ at 0.026 µM for *P. falciparum* and was confirmed inactive against *P. vivax* when assayed at a single concentration of 10 µM⁴⁵.

Next, a set of 913 repurposed compounds from the Caliber Bioactive Library were tested at 10 µM single concentration in the radical cure treatment mode. Compounds with schizontidal or hypnozonticidal activity were identified, including one compound, monensin, showing complete activity against both forms...
Fig. 7 Medium throughput dose response and single point antimalarial compound screening. 

**a** Dose response charts of inhibition of *P. vivax* and *P. falciparum* LS development following treatment with control compound KDU691 (a PI4K inhibitor) and reference compound atovaquone. 

**b** Dose response curves for six MMV portfolio drugs against both *P. vivax* and *P. falciparum* LS schizonts.

**c** Activity of 913 Calibr Bioactive compounds against *P. vivax* in radical cure mode; activity for each compound is indexed on the X axis by plate position with individual plate control wells grouped and indicated. Monensin, active against *P. vivax* hypnozoites and schizonts in both prophylactic and radical cure modes, is indicated within a gray circle.

**d** Dose response plots of inhibition of *P. vivax* LS parasites following treatment with three different ionophores or primaquine; PHH nuclei counts, normalized to DMSO only in lots with higher CYP2D6 activity (Supplementary Table 2, *P* = 0.013 for primaquine and MMV390048, a PI4K inhibitor, were found similarly active across all donors, but prodrug primaquine was found active only in lots with higher CYP2D6 activity (Supplementary Table 2, Supplementary Fig. 10).
Discussion

The lack of efficient high-throughput screening assays has been a major constraint limiting development of much needed new therapies targeting *P. vivax* and *P. falciparum* PE stages. The in vitro liver model reported here removes this barrier by significantly reducing the use of precious biological materials; each individual assay unit requires only 1.0 $\times$ 10^4 hepatocytes and only 5.0 $\times$ 10^4 *P. vivax* or *P. falciparum* sporozoites, representing a >20-fold reduction per assay unit relative to current platforms^{29,30,32}. When extrapolated for a full 384-well plate run at least 2.0 $\times$ 10^6 sporozoites are required per plate, a practical number to obtain from dissection of salivary glands from 20 to 200 mosquitoes. Nonetheless, this small cell culture surface area of only 10 mm^2 typically supported >200 *P. vivax* or >50 *P. falciparum* LS parasites per well, exceeding the averages of infected hepatocytes obtained in traditional models using larger 96-well plates^{29,30,32}.

We attribute our improved sporozoite isolation and infection techniques, as well as access to properly cryopreserved hepatocytes as the key factors responsible for the greatly improved infection rates. Following characterization of 13 different PHH lots in addition to lot PDC and HepaRG, several lots from were found highly viable, monolayer-forming, and functional; all characteristics ideal for *Plasmodium* LS development (Supplementary Tables 2 and 3, Supplementary Fig. 5). However, the specificity of PHH lots for either *P. falciparum* or *P. vivax* may be better attributed to the hepatic phenotypes. Of all the PHH donor lots examined for sporozoite invasion and LS development, some lots were not supportive of either or both *P. vivax* and *P. falciparum*. We hypothesize this bimodal support of infection in otherwise healthy hepatocytes is a product of natural variation in presentation of hepatic surface receptors CD81 and SR-BI, as *P. vivax* and *P. falciparum* hepatocyte entry has been found receptor-specific^{61}. While relative expression of surface receptors could not be analyzed in this study, additional proteomic and transcriptomic tools may be needed to identify biomarkers linked to a compatible donor phenotype. Alternatively, this variation could result from the cryopreservation process possibly leading to hepatocyte membrane receptor loss or undermining hepatic fitness to support intracellular parasites. Our hepatocyte bio-image assessment of the different PHH donor lots did suggest a strong link between enhanced sporozoite invasion and LS development to highly dynamic mitochondrial activity and increased bile canaliculi network formation. This indicates that the quality of hepatocyte lot may be more important than variation among individual donors. The mitochondria in hepatocytes are essential for survival, with main roles in energy metabolism (ATP), ion homeostasis, and apoptosis. Hepatic mitochondrial activity is likely vital in LS development, but host-parasite interactions are still largely uncharacterized and further investigation is needed^{62,63}. Regardless, we demonstrate the advantage of using a pre-validated, effectively cryopreserved PHH lots over cell lines as PHHs support higher invasion and development rates and full LS development during long-term radical cure vaccine and drug assays. Until more rapid phenotype assessment tools are available, investigators using PHHs for LS assays will first need to screen multiple lots to find the ones most suitable for their species of interest. Fortunately, with up to 1000 aliquots of cryopreserved PHHs resulting from a production run, many thousands of individual assay units can be experimentally analyzed with the same genotypic and phenotypic background of a single suitable hepatocyte lot after identification.

Equally important to reducing the rate-limiting requirements were mitigating costs and availability of materials and automation. We estimated the cost of consumable reagents (plate, hepatocytes, medium) were less than US ~$2 per well and materials could be readily shipped to assay locations. The live portion of the assay required no robotic automation, enabling rapid implementation of the protocol at multiple laboratories including those in malaria-endemic countries. This flexibility was and will continue to be essential for developing new antimalarial PE therapies, especially against vivax malaria, since blood stages of *P. vivax* cannot be continuously cultured making widespread production of gametocytes for PE assays in nonendemic countries a major obstacle^{65}. While shipment of *Plasmodium* spp.-infected mosquitoes from endemic countries to screening facilities is possible and was performed to generate some of these data described here, we note far greater throughput can be achieved by moving the assay itself to the endemic country to be supported by sporozoites produced from local infections. As malaria control increases worldwide, continued efforts to screen for PE therapeutics could be enabled by cryopreserved sporozoites, a concept also demonstrated in this study (Fig. 3a, Supplementary Fig. 4a, b, and Supplementary Fig. 9). Although direct comparisons were not made, our results surprisingly suggest laboratory strains of *P. falciparum* have reduced fitness in an in vitro LS assay.

The staining patterns with the panel of immuno-reagents used to characterize the LS morphologies in our in vitro cultures were similar to that noted in in vivo humanized mouse models, with either *P. vivax* or *P. falciparum*, confirming complete LS development in vitro^{31,32}. In both mice and our in vitro culture, LS schizonts exhibit several large circular vacuoles of regular size, each about 1/20–1/10 the parasite total 2D area as observed under microscopy. The role and contents of these vacuoles are unknown, but high-resolution imaging revealed the membrane limiting the vacuole contained EXP2 and not UIS-4 and the internal void lacked MIF. Based on these observations, these uncharacterized vacuoles are likely a formation of the parasite membrane and not the parasitophorous vacuole membrane and would be separate from the parasite’s cytoplasm. As *Plasmodium* biology and morphology contains several well-characterized sequestration mechanisms (i.e., micronemes and dense granules for invasion protein machinery of sporozoites and merozoites, or the digestive vacuole for sequestration of toxic hemoglobin produced during the erythrocytic stage), further characterization of the contents in these novel LS compartments could reveal interesting new drug targets such as resident solute pumps or possible toxic byproducts produced during maturation^{66}. Morphological characterization also helps explain one of the unknowns of *P. vivax* lifecycle—the parasite is able to form infectious gametocytes in only days compared to over 2 weeks for *P. falciparum* and epidemiology studies show gametocytes are circulating within days of LS development^{64,65}. We find a large proportion of completely mature *P. vivax* LS schizonts express gametocyte commitment marker Pvs16 such that all merozoites in the form are positive; while the remaining schizonts are negative for Pvs16. As commitment to gametocytogenesis has been described to begin in the asexual lifecycle producing the merozoites that will form a gamete, this stain pattern suggests whole sub-populations of *P. vivax* LS schizonts are producing gamete-forming merozoites instead of asexually replicating merozoites^{66}. This mechanism, if confirmed, would help explain vivax transmission between an asymptomatic individual and the mosquito vector.

Key to the development of the next generation antimalarial toolbox, our platform is the first to perform extended ILSDA’s (days 6 or 8) in PHHs revealing not only inhibitory effects but also growth attenuation of maturing *P. falciparum* and *P. vivax* LS parasites^{67}. Our ILSDA offers solutions to inherent issues of traditional ILSDA methods through assay standardization and high infection rates, thus greatly increasing reproducibility and sensitivity^{45,50,68}. We show immune serum raised against SAPN-produced antibodies to FMP014/ALF (G2) and FMP014V/ALF (G6) both inhibit invasion of *P. falciparum* sporozoites. This
finding is of high importance as FMP014V/ALF does not have PICSP NAP repeats, indicating all inhibition comes from antibodies to the α-TSR region, thus potentially leading to longer-lived levels of protection55. Lastly, our model has the ability to screen late-stage-arresting genetically-attenuated sporozoite vaccines along with vaccine candidates targeting first generation LS merozoites entering into blood-stage. Medium throughput small molecule screening was performed using our HCl endpoint to quantify the number and growth of LS schizonts and differentiate P. vivax schizonts from hypnozoites. Control compounds atovaquone and KDU691 behaved as expected following characterization of these compounds in vitro and in vivo26,70. We also confirmed cross-species activity of several preclinical candidate drugs to characterize the predictive value of our screen. To better understand the possible effect of host-specific metabolism, an assessment of compound efficacy in different PHH lots found no drastic PHH-specific differences for optimized compounds, including MMV390484 and atovaquone, but did find a lot-specific outcome with primaquine in relation to each lot’s CYP2D6 activity and these lots’ ability to produce the active primaquine metabolite (PQ+; directly quantified). In addition to differential host cell metabolism, measurement of primaquine efficacy is confounded by how quickly treated LS parasites clear from culture. Primaquine treatment in vivo has been shown to result in malformation, but not necessarily clearance, of LS parasites, while our radical cure assay was taken out to only 3 days post compound addition so as not to miss observation of schizonticidal compound activity70. To alleviate concerns of a false negative due to metabolism, dosing concentration and repetitions (maximum 10 µM administered 3 times) were optimized to provide excessive opportunity for metabolically labile compounds to act on LS parasites. Furthermore, the MMV compounds and Calib Bioactive Library screened were all metabolism-optimized compounds, thus reducing the likelihood of a false negative due to metabolism. Despite these protocol adaptations to circumvent hepatic metabolism, we cannot rule out that our screen may miss quickly metabolized compounds. Application of our model to single point screening revealed several interesting hits, including the ionophore monensin, and suggests P. vivax schizonts and hypnozoites are sensitive to ion homeostasis. The ionophores will be best used as positive controls for radical cure assays rather than new drugs given the known problems with ionophore toxicity and poor selectivity index71. In the least, these hits demonstrate dead LS parasites can be cleared from PHH culture, and therefore drug activity can be detected, following a relatively short 3-day treatment. These ionophore hits also suggest ion homeostasis could be a viable target for anti-malarial drug discovery72.

Hepatocytes functional assays. Albumin and Factor IX production were measured by Bioanalysis IVT (BIVT) from hepatocyte (PHH) or hepatoma (HepaRG) cells treated with 25 µM Rifampicin (Cat No. BP26795, Fisher, Hampton, NH, USA) in plate media, or an equivalent volume of DMSO in unduced controls, and luciferin signal measured by a luminance plate reader following the lytic version of the manufacturer’s protocol. Basal enzymatic characterization was performed for all 14 BIVT donor lots and provided by Bior- eclamation IVT (Inc (Baltimore, MD, USA)). Hepatocyte functional assays were induced with 25 µM Rifampicin for 72 h, with 25 µM Rifampicin for 24 h and then storing samples at −80 °C until collection was completed. For albumin measurements, 12 wells (at 50 µl volume) were collected every 2 days with 30 days with media addition after time point removal. Albumin measurements (ng · m−2) were collected using the Albumin (human) AlphaLISA Detection kit specific for the human albumin (Cat No. V1122-A) on a luminometer (Perkin Elmer) and the mean with s.d. (n = 6) were reported. Samples (n = 3) were quantified for Factor IX ELISA per manufacturer’s protocols (Cat No. EF1009-1, AssayPro, St. Charles, MO, USA). Cytochrome P450 3A4 induction was measured using CYP3A44 P450 Glo luciferin-IPA kit (Cat No. V9002, Promega, Madison, WI, USA). Hepatocytes were induced for 72 h with 25 µM Rifampicin (Cat No. BP26795, Fisher, Hampton, NH, USA) in plate media, or an equivalent volume of DMSO in unduced controls, and luciferin signal measured by a luminance plate reader following the lytic version of the manufacturer’s protocol. Basal enzymatic characterization was performed for all 14 BIVT donor lots and provided by Bior- eclamation IVT (Inc (Baltimore, MD, USA)). Metabolic activity was calculated and results represent the average of the 3 samples (Supplementary Table 2 and Supplementary Fig. 5). Activity results were analyzed by HPLC-UV or LC/MS/MS validated procedures. Metabolite formation for all enzymes was measured after a 60 min. Cell incubation at 37 °C, 5% CO₂ resulting in a final concentration of 1 million cells per ml. The standard 60 min incubation was used for this assay.

Live imaging and analysis of hepatobiliary formation. A total of 6 time points (day 2, 4, 8, 14, 21, 30) with experimental replicate wells (n = 3, n = 3) were selected to phenotypically assay the PPHs (donor lot PDC) seeded in the commercial 384-well plate (Fig. 2a). For the Bioanalysis IVT PHH donor screen, a total of 4 time points (day 2, 4, 8, 14) with experimental replicate wells (n = 1, n = 3) were selected to phenotypically assay the PPHs seeded in the commercial 384-well plate (Supplementary Table 3). Wells were stained with 25 µM Mg Lactoplasmin (Promega, Madison, WI, USA) and 10 µg/ml of a live imaging agent CellTracker Green CMFDA dye (Cat No. C7025, Life Technologies, Carlsbad, CA, USA) to capture hepatocyte bile canaliculi formation. At 48 h post-seed, images were collected every 24 h for 30 days with imaging machine (Perkin Elmer, Waltham, MA, USA) were used in wide field mode (>20 objective, 0.4 NA) to capture 35 tiles within each 384-well while simultaneously acquiring 4 channels (Transmitted, TRITC, FITC, DAPI) representing brightfield, respiration, transport, and viability. A z-plane stack of 2–10 µm with a 2 µm plane distance was utilized for each well. Using Z-stacking, a maximum intensity image projection merging all z-planes was formatted for each well per time point. First, total hepatocytes per well were calculated by using Hoechst staining of hepatocyte nuclei where populations termed “healthy” and “unhealthy” were determined by area (µm²) and MFI ≥ 3000. Next, the area and fluorescent intensity of TMRM was calculated using the emitted fluorescence (TRITC channel) where the MFI of entire well was used to compare mitochondrial activity between the time points. To accurately find and classify the bile canaliculi as active or inactive transport, a combination of several building blocks and image filter sets were required using Harmonie software (Harmony software (4.1) (Supplementary Fig. 3). Post compilation of the z-stack, a Gaussian image filter was applied to enrich contrast of active bile canaliculi and normalize background intensity. Simultaneously, the image was filtered for intensity of the FITC channel allowing for identification of hepatocyte cells incapable of transporting dye. An image calculation was applied (Gaussian filtered intensity thresholding of TRITC channel) for all classified bile canaliculi at all time points; morphology (length, width, area, roundness), and intensity (sum, mean, minimum, maximum). Thresholds to

Methods

Seeding 384-well plates with human hepatocytes. Individual wells of a 384-well plate (Cat No. 781091, Greiner, Monroe, NC, USA) were coated with rat tail collagen I (Cat No. 534236, Corning, New York, NY, USA) diluted to a final concentration of 5 µg collagen per cm² in 0.02 M acetic acid, incubated at 37 °C overnight to ensure adsorption and washed thrice with 1 × PBS. Alternatively, after experimental confirmation, pre-coated collagen plates (Cat No. 781956, Greiner, Monroe, NC, USA) were used for all compound screening and a selection of ILSDA studies. For ILSDA and drug studies, cryopreserved primary human hepatocytes (Cat No. M00995-P and F00995-P, donor lots PDC, UBV, NLX) and hepatocyte culture medium (HCM) (InVitroGroTM CP Medium) were obtained from Bio-reclamation IVT, Inc (Baltimore, MD, USA). For donor screens, cryopreserved primary human hepatocytes were obtained from four companies (Cat No. M00995-P, donor lots PDC (Harmony IVT, U.S.A), (Cat No. HU3CD, donor lots HUM40966, HUM4074D, Triangle Research Labs LLC, Durham, NC, USA). (Cat No. 20-0047, donor HHHF17006 lot RTA0006 and donor HHH01013 lot DFA0003, Yeucurs), (TUAT, Tokyo, USA), (donor lot HW-40, HW39, no label, Mass General Hospital, Boston, MA, USA) and maintained following manufacturer’s protocols. For HepaRG studies, NoSpin HepaRGM™, a commercially available, terminal-differentiated hepatic cell line, were seeded at 20,000 liver hepatocytes per well following the manufacturer’s instructions (Cat No. NSHPRG, Triangle Research Labs LLC, Durham, NC, USA). Hepatocytes were supplemented with a final concentration of 10 µg/ml of penicillin-streptomycin, 100 µg/ml of neomycin, and 10 µg/ml of gentamicin. Cultures were kept on HCM (media containing serum) throughout studies as serum-containing media was found important for long-term viability. Cryopreserved PHHs were thawed following manufacturer’s protocols and viability assessed by trypan blue exclusion (typically 70–99%), resulting in recovery of 5–9 million viable cells per vial. Thawed PHHs were then diluted in HCM to a concentration of 800–900 cells per µl with 20 µl cell suspension added to individual wells. The relative number of hepatocytes seeded per well was set to achieve a confluent monolayer. At 2 days post seed each well received a complete media change (40–50 µl), then media was changed per the experimental protocol (Fig. 1b).

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P. falciparum mosquito infections from in vitro culture. P. falciparum (strain NF54) was maintained according to standard methods at 37 °C (5% O2 and 5% CO2, nitrogen balanced) in 5% hematocrit (O + blood), 10% AB human serum (The Interstate Blood Bank INC, Memphis, TN, USA), RPMI 1640 medium (Cat No. CUS-0645, KD medical, Columbia, MD, USA), and 2.5% sodium bicarbonate (Sigma, St. Louis, MO, USA)26.

P. falciparum sporozoite infections from infected mosquitoes. Mosquitoes 14–16 days after infection were aspirated dissected and collected into Schneider’s media (Cat No. S9895, Sigma-Aldrich, St. Louis, MO, USA)27.

Ethical statement. The Human subjects protocols for this study was approved by the Institutional Ethics Committee of the Thai Ministry of Public Health, the Human Subjects Sub-committee of the Review Board of the U.S. Army (WRAIR/4049), the Ethical Review Committee of Faculty of Tropical Medicine, Mahidol University (TMEC-11-008 and 14-016), the Oxford Tropical Medicine Ethical Committee, Oxford University, England (OxTREC-17-11 and 40-14), and the Cambodian National Ethics Committee for Health Research (10/NECHR). The protocols conformed to the Health Canada’s Ethical principles for research involving human subjects (version 2002) and informed written consent was obtained for all volunteers.

P. vivax mosquito infections from infected patients. Isolates of P. vivax were collected from symptomatic patients enrolled in the study after giving written informed consent at the Thai malaria clinic (SMRU) in Tak province, Mae Sod malaria project (Thailand Ministry of Public Health) in Tak province, recruitment via the village malaria workers network and the local health centers in the Mundokiri Province in Cambodia. A sample of P. vivax-infected blood was drawn by venipuncture into heparin tubes. Thick and thin Giemsa-stained smears were prepared and submitted to the mature and infected stages were stored in a −80 °C freezer. Sporozoites were centrifuged at 200 × g for 5 min. Drug regimen was defined before transfer, prophylactic or radical cure, with addition of drug on assigned days and HCM supplemented with anti-microbials replenished daily. Compounds were then dissolved in 100% DMSO to stock concentrations of 10 mM and then dispensed and serially diluted in absorption 384-well master plates (Axxygen, Tewksbury, MA, USA) using a multichannel pipette. Plated compounds were then stored at −20 °C and after shipment to an endemic laboratory (SMRU in Thailand and IPC in Cambodia) on dry ice. Plated compounds were thawed at room temperature, spun down at 200 × g for 5 min and stored covered with foil tape in an ambient desiccation chamber between daily transfers. Transfer of compounds from the master plate to the 384-well plate was performed using a sterilized manual pipette tool (VP Scientific, San Diego, CA, USA) engineered to dispense 40 µl of liquid per pin into 40 µl HCM volume in the well to achieve a 1:100-fold dilution. Before and after compound transfer the pin tool was cleaned by two wash cycles (dip and blot) in each of 50% DMSO in water, then 70% EtOH in water, then VP clean solution, then 100% EtOH, per manufactured instructions. Initially, compounds were tested in independent experiments using an 8-point concentration format with 3-fold dilutions (final concentrations of 10 µM to 5 nM). For Single Point assays, compounds were tested and the most potent 24 hits were selected for confirmation and EC50 determination in the same 8-point concentration format described above. For each assay plate, both a positive standard at 100 µM (KDU691) and negative (0.1% DMSO) control were added and a minimum of 8 wells per plate were analyzed. Based on controls, a Z′ factor was calculated for each plate and positive value were considered acceptable for data analysis28.

Automated image acquisition and data analysis. At 5–8 days post infection, wells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature and washed twice with 1×PBS. For P. falciparum experiments, wells were incubated in blocking buffer (0.03% Triton X-100 in 1×PBS) for 1 h before adding anti-GADPH (3.2 µg ml−1, 1:5000 dilution) overnight at 4 °C. For P. vivax experiments, wells were incubated in blocking buffer with polyclonal rabbit or mouse recombinant anti-UlUS mAb (1 µg ml−1, 1:5000 or 1:25,000-fold dilution) overnight at 4 °C. After, the wells were washed thrice with 1× PBS and incubated for 1 h at room temperature with either goat anti-rabbit or goat anti-mouse Alexa Fluor® 488 conjugate (2 µg ml−1, 1:1000-fold dilution) or at 1:1000-fold dilution) then washed thrice, and filled with 1×PBS for imaging and storage.

Infection with cryopreserved Plasmodium sporozoites. Previously cryopreserved lots of P. vivax and P. falciparum sporozoites were used to assess PHH invasion and development. Cryopreservation of sporozoites were performed following previously published protocols and thawed at room temperature with dilution in HCM29. A lot of cryopreserved sporozoites refers to a single patient derived case while a lot of cryopreserved P. falciparum sporozoites refers to lab-adapted strain NF54. A cryopreserved P. vivax sporozoite lot was used and infected at 2.0 × 104 sporozoites per well (Supplementary Fig. 4a, b, Supplementary Fig. 9, and Supplementary Table 1) or at 1.0 × 104 sporozoites per well (Fig. 3a and Supplementary Table 3). A cryopreserved P. falciparum sporozoite lot was used and infected at 2.5 × 104 sporozoites per well (Fig. 3a and Supplementary Table 3).

Inhibition of Liver Stage Development Assays (ILSDAs). After salivary gland dissections at WRAIR, SMRU, IPC, or USF, sporozoites were counted using a hemocytometer and diluted accordingly in HCM. For antibody and serum exposure, sporozoites were incubated with test serum samples in a serially diluted manner (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 in HCM) or with monospecific antibody microwell spotted (10, 20, 5, 1, 0.25, 0.125 µg ml−1 in microwell) at 250, 25, 2.5, 0.25 µg ml−1 (P. vivax) for 20 min at room temperature or overnight at 37 °C, 5% CO2. For experimental control, sporozoites were exposed to pre-blend mouse sera as well as in absence of an exposure (HCM only) then held at room temperature for 20 min or overnight at 37 °C, 5% CO2. After incubation, 1.0 × 104 sporozoites from each condition were added into duplicate wells, spun down at 200 × g for 5 min, and allowed to invade for 24 h at 37 °C before washing with HCM. Media was changed every 2 days using HCM supplemented with antimicrobial mix until fixation 6 days post initiation.
IgG curves and % inhibition of ILSDAs was performed using dose-response modeling in Prism (Graphpad, La Jolla, CA, USA) where data were normalized to the negative control (injected well, ICtrl) described by the equation below:

\[
\% \text{Inhibition} = 100 - \left( \frac{X}{\text{ICtrl}} \right) \times 100
\]

where X is the measured inhibition by the antibody or immune sera and ICtrl is calculated using the average of 12 experimental replicates in the same 384-well plate.

High-resolution imaging and blood breakthrough assays. Briefly, the same immunofluorescence assay (IFA) protocol was followed above with the following fold dilutions of monoclonal antibodies obtained from The European Malaria Reagent Repository: mouse anti-GAPDH (Cat No. 7.2) at 1:50,000, mouse anti-EXP1 (Cat No. A20186) at 1:100,000, mouse anti-GLURP (clones 2C26, 8B12, 2C7) at 1:200, -anti-MSP1 (Cat No. 12.10) at 1:2,000, and -anti-MSP2 (Cat No. 12.3) at 1:2001–4. The following antibodies were used; polyclonal rabbit anti-USP-4 at 1:5,000, recombinant mouse USP-4 at 1:25,000, polyclonal rabbit anti-MIF 1:1,000, and monoclonal mouse ACP at 1:1,000 and were obtained from Center for Infectious Disease Research, WA, USA15. The anti-USP4 recombinant monoclonal antibody used in this study was derived from a hybridoma expressing anti-USP4 monoclonal antibody developed by methods previously described16,17. We pro-duced recombinant mouse anti-Pvs16 then conjugated to Alexafluor® 647 and used experimental dilutions at 1:50 (Cat No. A20186, Molecular Probes, ThermoFisher, Waltham, MA, USA). We also produced a mouse anti-HSP70 hybridoma supernatant (clone 4C9) and used at 1:100. After primary staining, the wells were washed thrice with 1× PBS and incubated for 1 h at room temperature with either goat anti-rabbit Alexa Fluor® 488 conjugate, goat anti-mouse Alexa Fluor® 488 conjugate, goat anti-rabbit Alexa Fluor® 568, or goat anti-mouse Alexa Fluor® 568 conjugate (2 µg ml−1, 1:1,000-fold dilution; Cat No. A11001, A11034, A11004, A11011, Molecular Probes, ThermoFisher, Waltham, MA, USA) secondary anti-body and Hoechst (10 µg ml−1, 1:1,000-fold dilution) then washed thrice, and filled with 1× PBS for imaging and storage. High-resolution, z-stacked images of P. vivax and P. falciparum LS parasites were captured with a ×100 oil objective, 1.4 NA on a DeltaVision Core system (GE Healthcare Life Sciences, Piscataway Township, NJ, USA). Images were deconvoluted using the softWoRx® image analysis package (GE Healthcare Life Sciences, Piscataway Township, NJ, USA) and z-stacks with added scale bars were processed in FIJI31. Images captured with secondary antibody Alexa Fluor® 568 were re-colored to magenta using FIJI in order to meet publication image standards.

Plasmodium blood breakthrough studies were performed using either fresh reticulocytes prepared from Duffy antigen positive buffy packs (P. vivax) or fresh O + whole blood (The Interstate Blood Bank INC, Memphis, TN, USA). O + blood (25 ml) was centrifuged at 2000 RCF for 5 min to remove Buffy coat and RBCs were washed thrice with 1× PBS and incubated for 1 h at room temperature with either goat anti-rabbit Alexa Fluor® 488 conjugate, goat anti-mouse Alexa Fluor® 488 conjugate (2 µg ml−1, 1:1,000-fold dilution; Cat No. A11001, A11034, A11004, A11011, Molecular Probes, ThermoFisher, Waltham, MA, USA) secondary anti-body and Hoechst (10 µg ml−1, 1:1,000-fold dilution) then washed thrice, and filled with 1× PBS for imaging and storage. High-resolution, z-stacked images of P. vivax and P. falciparum LS parasites were captured with a ×100 oil objective, 1.4 NA on a DeltaVision Core system (GE Healthcare Life Sciences, Piscataway Township, NJ, USA). Images were deconvoluted using the softWoRx® image analysis package (GE Healthcare Life Sciences, Piscataway Township, NJ, USA) and z-stacks with added scale bars were processed in FIJI31. Images captured with secondary antibody Alexa Fluor® 568 were re-colored to magenta using FIJI in order to meet publication image standards.

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