Asparagine requirement in *Plasmodium berghei* as a target to prevent malaria transmission and liver infections

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The proteins of *Plasmodium*, the malaria parasite, are strikingly rich in asparagine. *Plasmodium* depends primarily on host haemoglobin degradation for amino acids and has a rudimentary pathway for amino acid biosynthesis, but retains a gene encoding asparagine synthetase (AS). Here we show that deletion of AS in *Plasmodium berghei* (*Pb*) delays the asexual- and liver-stage development with substantial reduction in the formation of ookinetes, oocysts and sporozoites in mosquitoes. In the absence of asparagine synthesis, extracellular asparagine supports suboptimal survival of *Pb*AS knockout (KO) parasites. Depletion of blood asparagine levels by treating *Pb*ASKO-infected mice with asparaginase completely prevents the development of liver stages, exflagellation of male gametocytes and the subsequent formation of sexual stages. In vivo supplementation of asparagine in mice restores the exflagellation of *Pb*ASKO parasites. Thus, the parasite life cycle has an absolute requirement for asparagine, which we propose could be targeted to prevent malaria transmission and liver infections.
Malaria, transmitted through female Anopheles mosquito bites, is a devastating disease that causes close to 0.6 million deaths annually. Of the five Plasmodium species that cause human malaria, Plasmodium falciparum and Plasmodium vivax infections are the most common. While 90% of the malaria-associated deaths occur in Africa due to P. falciparum infections, P. vivax infections are prevalent in Southeast Asia and South America. With the absence of a highly effective vaccine and with widespread resistance to known antimalarials such as chloroquine and antifolates, artemisinin-based combinations have remained as primary options for malaria therapy1–3. However, the emergence of resistance to artemisinin derivatives and various partner drugs such as amodiaquine, mefloquine and piperaquine has been a matter of grave concern4–7. Besides asexual stages for which haemoglobin and hemozoin assimilatory pathways have served as major drug targets8–10, malaria eradication would also demand targets to prevent transmission and liver infections5. Also, attempts are underway to develop drugs that can offer single-exposure radical cure, prophylaxis and chemoprophylaxis11,12. Thus in turn underscores the need to identify new domains of drug targets that would provide therapeutic options for targeting multiple stages of the parasite cycle.

One such versatile target would be amino acids—molecular building blocks of proteins, precursors of various biologically important molecules and essential sources of carbon, nitrogen and energy metabolism13,14. Plasmodium lacks the canonical pathways for amino acid biosynthesis15,16 and, therefore, relies mainly on host haemoglobin degradation and extracellular sources to meet its amino acid requirements. Studies carried out using minimal RPMI medium devoid of amino acids have revealed that the blood-stage parasites can survive and proliferate when isoleucine—the only amino acid that is absent in amodiaquine, mefloquine and piperaquine has been a matter of grave concern4–7. Besides asexual stages for which haemoglobin and hemozoin assimilatory pathways have served as major drug targets8–10, malaria eradication would also demand targets to prevent transmission and liver infections5. Also, attempts are underway to develop drugs that can offer single-exposure radical cure, prophylaxis and chemoprophylaxis11,12. Thus in turn underscores the need to identify new domains of drug targets that would provide therapeutic options for targeting multiple stages of the parasite cycle.

One such versatile target would be amino acids—molecular building blocks of proteins, precursors of various biologically important molecules and essential sources of carbon, nitrogen and energy metabolism13,14. Plasmodium lacks the canonical pathways for amino acid biosynthesis15,16 and, therefore, relies mainly on host haemoglobin degradation and extracellular sources to meet its amino acid requirements. Studies carried out using minimal RPMI medium devoid of amino acids have revealed that the blood-stage parasites can survive and proliferate when isoleucine—the only amino acid that is absent in haemoglobin—is provided as a sole exogenous amino acid15,16. This indeed exemplifies that the amino acids derived from haemoglobin degradation are adequate to support the growth when blood-stage parasites are supplemented with isoleucine. Although auxotrophic for most of its amino acids, the parasite genome encodes a few enzymes synthesizing glycine, proline, glutamate, glutamine, aspartate and asparagine, the functional significance of which remains unknown15,16. Of these amino acids, asparagine plays a pivotal role in the parasite life cycle by serving as one of the most abundant amino acids in Plasmodium proteins18. While this could be partly explained by the A–T rich nature of Plasmodium genomes, the frequency of asparagine is also quite high in the proteins of P. vivax and P. knowlesi whose genomes are relatively G–C rich18–20. Consequently, the malaria parasite has also retained asparaginase synthetase (AS) that catalyses the formation of asparagine from aspartate.

AS is an important enzyme as a chemotherapeutic target for acute lymphoblastic leukaemia (ALL)21. Since leukaemia cells depend primarily on exogenous asparagine for their proliferation, asparaginase treatment has been successfully carried out in patients with ALL to deplete the malignant cells from asparagine22,23. Also, there are efforts to develop AS inhibitors that can circumvent the development of resistance towards asparaginase treatment that has been mainly attributed to the upregulation of endogenous AS in leukaemia cells21,24. Given the proliferating ability of malaria parasites and their analogy to cancer cells together with the abundance of asparagine in the parasite18,25, it would be interesting to explore the asparagine requirement in malaria parasites as a therapeutic target.

Amino acid requirements in the malaria parasite have been hitherto considered only in the blood stages where haemoglobin serves a major reservoir of amino acids17,26–28. Here we use P. berghei as an in vivo rodent parasite model to address the significance of asparagine requirement in the entire life cycle of malaria parasites by performing targeted deletion of endogenous AS and depleting the extracellular asparagine by asparaginase treatment. We show that extracellular asparagine plays a key role in PbAS knockout (PbASKO)-infected mosquitoes and mice. We propose a combined approach of targeting the endogenous AS and depleting the extracellular asparagine to prevent transmission and liver infections. To the best of our knowledge, this is the first report emphasizing the significance of amino acid requirements and the contribution of extracellular sources in the sexual and liver stages where the parasite development takes place in a haemoglobin-free environment. Our study substantiates the therapeutic potential of amino acid requirements for targeting the multiple stages of malaria parasite life cycle.

Results

P. berghei AS is enzymatically active. Asparagine biosynthesis is catalysed by two evolutionarily independent families of enzymes—AS-A and AS-B25,30—of which AS-A catalyses the amidation of aspartate using ammonia as nitrogen source, whereas AS-B can utilize both glutamine and ammonia although glutamine is preferred under physiological conditions (Fig. 1a). Sequence analysis of the annotated Plasmodium AS revealed that the parasite enzyme belongs to AS-B, comprising an N-terminal glutamine-hydrolyzing domain representing class-II glutamine amidotransferases/N-terminal aminohydrolases superfamily and a C-terminal domain representing ATP pyrophosphatases/adenine nucleotide alpha hydrolases superfamily21. The ammonia released during glutamine hydrolysis traverses an intramolecular tunnel and reaches the active site of C-terminal domain where it reacts with β-aspartyl-AMP intermediate to form asparagine31. The critical residues identified based on the crystal structure of Escherichia coli AS-B such as Cys1, Arg49, Leu50, Ile52, Val53, Asn74, Gly75, Glu76 and Asp98 (required for positioning and hydrolysis of glutamine), Ser234, Gly235, Gly236, Leu237 and Asp238 (constituting the ATP pyrophosphate loop motif) and Leu232, Ser346, Gly347, Gly352, Tyr357, Lys376, Asp384, Arg387 and Lys449 (interacting with β-aspartyl-AMP intermediate; numbers representing E. coli AS-B without N-terminal methionine)21,31 were found to be well conserved in Plasmodium AS (Fig. 1b).

To confirm that the parasite enzyme possesses AS-B activity, PbAS cDNA was cloned into pET-20b (+) plasmid (Supplementary Fig. 1a) and the C-terminal His-tagged PbAS was over-expressed in E. coli Rosetta2DE3Plys strain and purified using Ni²⁺-NTA resin (Fig. 1c). Since N-terminal His-tag fusion did not yield an active enzyme, pET-20b (+) was chosen to overexpress the recombinant PbAS with C-terminal polyhistidine tag. In western blot analysis, the purified recombinant PbAS (67 kDa) was reactive with both anti-polyHistidine (Sigma-Aldrich) antibody (Fig. 1d) and anti-PbAS IgG (Fig. 1e) purified from polyclonal serum-raised against the recombinant protein. AS-B is known to exist as a homodimer and this seems to be essential for its catalytic activity21. To confirm the homogeneity of purified recombinant PbAS and rule out the possibility of PbAS forming a hybrid dimer with E. coli AS, matrix-assisted laser desorption/ionization analysis of purified PbAS was carried out in which the tryptic peptides corresponding only to PbAS, but not E. coli AS were detected (Supplementary Fig. 1b). Kinetic studies carried out with recombinant PbAS using a coupled assay system32 indicated that the parasite enzyme can more efficiently synthesize asparagine when glutamine was provided as an amino group donor. The Kₘ value for aspartate and the specific activity of PbAS were found to be 0.26 ± 0.05 mM and 0.79 ± 0.03 μmol mg⁻¹ min⁻¹, respectively, with glutamine as amino donor (Fig. 1f). The corresponding values for aspartate
Asparagine synthetase of malaria parasites exhibits AS-B activity. (a) Schematic representation of AS-A (ammonia dependent) and AS-B (both glutamine and ammonia dependent) activities. (b) Structure-based sequence alignment of P. berghei AS (Plasmodium) with E. coli and human AS-B. α-helices and β-sheets are represented in red and blue, respectively. N-terminal domain (1–202 residues) representing Class-II glutamine amidotransferases/N-terminal aminohydrolases (Ntn) superfamily and C-terminal domain (203–581 residues) representing ATP pyrophosphatases/adenine nucleotide alpha hydrolases superfamily are highlighted in boxes. The residues conserved across all three ASs are marked by asterisks (*). (c) SDS–PAGE analysis of purified recombinant PbAS (67 kDa). Lane M: Protein molecular weight marker (kDa). (d,e) Western analyses of recombinant PbAS with anti-polyHistidine antibody (d) and anti-PbAS IgG (e), respectively (full blots are shown in Supplementary Fig. 6). Lane M: Prestained protein molecular weight marker (kDa). (f,g) Lineweaver-Burk plots for PbAS with either glutamine (f) or ammonia (g) as nitrogen source. The assays were carried out using different concentrations of aspartate. (h) Radioactive assays performed with [U-14C]-aspartate and their product identification by TLC. For control, heat-denatured enzyme was used.

with ammonia as amino donor were $1.12 \pm 0.12$ mM and $0.18 \pm 0.03$ μmol mg$^{-1}$ min$^{-1}$, respectively (Fig.1g). The catalytic efficiency of PbAS for aspartate in the presence of glutamine ($k_{cat}/K_m = 2.7 \times 10^3$ M$^{-1}$ s$^{-1}$) was found to be similar to that of human AS for which the reported $K_m$ and $k_{cat}/K_m$ values were $42.7 \pm 0.05$ mM and $3.4 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively$^{32}$. Given the constraints associated with coupled assay system in crude lysates, a simple radioactive assay with [U-14C]-aspartate was developed...
and the formation of radiolabelled asparagine was detected by thin-layer chromatography (TLC) as well (Fig. 1h). Similar results were also obtained with recombinant PfAS, which shares 77% identity and 86% similarity with PbAS (Supplementary Fig. 2). All these data clearly demonstrated that the AS of malaria parasite is enzymatically active.

**Targeted deletion disrupts the chromosomal locus of PbAS.** To determine the significance of AS in the parasite life cycle, chromosomal locus of AS was targeted through double cross-over recombination using pL0006 plasmid in which 5′- and 3′-untranslated regions (UTRs) of AS were cloned on either side flanking human DHFR selection cassette (Fig. 2a). Ten days post transfection, limiting dilution was carried out for pyrimethamine-resistant parasites to select the individual clones\(^{33}\). PCR and reverse transcriptase–PCR (RT-PCR) analyses performed with genomic DNA and RNA isolated from Pb wild-type (WT) and PbASKO parasites revealed the absence of 2.53 and 1.75 kb products in PbASKO suggesting the targeted deletion of PbAS locus (Fig. 2b,c). This was confirmed by Southern analysis wherein respective products of 3.4 and 2.0 kb were detected for PbWT and PbASKO parasites (Fig. 2d). Also, northern (Fig. 2e) and western (Fig. 2f) analyses were carried out to ensure the absence of AS mRNA (1.75 kb) and AS protein (67 kDa) in PbASKO. This was further substantiated with enzyme assays using \([U-^{14}C]\)-aspartate where the formation of asparagine could be detected in PbWT, but not in PbASKO parasites (Fig. 2g). Thus, the chromosomal locus of AS in *P. berghei* was successfully targeted to generate the KO parasites.

**Figure 2 | Generation of AS knockout in *P. berghei*. (a)** Double cross-over recombination strategy followed to generate PbASKO. (b) PCR analysis for genomic DNA isolated from PbWT and PbASKO parasites. Lane 1 and 3: PCR with PbAS-specific primers; Lane 2 and 4: PCR with PbGAPDH-specific primers (control); Lane M: 1 kb DNA ladder. (c) RT-PCR analysis for RNA isolated from PbWT and PbASKO parasites. Lane 1 and 3: RT-PCR with PbAS-specific primers; Lane 2 and 4: RT-PCR with PbGAPDH specific primers (control); Lane M: 1 kb DNA ladder. (d) Southern analysis for genomic DNA isolated from PbWT and PbASKO parasites. Transgenic plasmid (TP) was also included as control to rule out the presence of any episomes. Genomic DNA preparations and TP were digested with BsrGI and XbaI followed by hybridization with 5′-UTR specific probe. (e) Northern analysis for RNA isolated from PbWT and PbASKO parasites indicating the absence of AS mRNA (1.75 kb) in PbASKO. For control, GAPDH (1.01 kb; lower panel) was used (full blots are shown in Supplementary Fig. 6). (f) Western blot analysis indicating the absence of AS (67 kDa) in PbASKO parasite lysate (Supplementary Fig. 6). Parasite lysates of PbWT and PbASKO containing 100 μg of total protein were used. For control, hsp60 (60 kDa) was used (lower panel). (g) Enzyme assays for PbWT and PbASKO parasites using \([U-^{14}C]\)-aspartate. 200 μg of total protein was used per assay.
Asparaginase blocks PbsaskO sexual- and liver-stage formation. To examine whether extracellular asparagine is critical for the survival of PbsaskO parasites lacking endogenous asparagine synthesis, mice infected intraperitoneally with $10^5$ PbsWT or PbsaskO asexual-stage parasites were treated with three doses of L-asparaginase (50 IU per mouse) on day 5, 6 and 7. Liquid chromatography-selected reaction monitoring/mass spectrometry–based quantification of plasma asparagine (Supplementary Fig. 4) on day 8 revealed more than 90% reduction in the plasma asparagine levels of mice treated with asparaginase (Fig. 4a). However, the growth curves obtained for PbsWT and PbsaskO (Fig. 4b) intra-erythrocytic development indicated that there was only a slight and statistically insignificant (two-tailed unpaired Student’s t-test) delay of 1–2 days in overall survival of mice after asparaginase treatment (Fig. 4c). For sexual-stage development analyses, mosquito-feeding experiments were carried out on day 8 using asparaginase-treated mice. While asparaginase treatment per se did not affect the formation of sexual stages in PbsWT-infected mosquitoes, no ookinetes, oocysts or sporozoites could be detected in mosquitoes fed on PbsaskO-infected mice treated with asparaginase (Figs 4d–i). Even more interestingly, asparaginase treatment completely prevented the liver-stage development of PbsaskO sporozoites when $10^4$ sporozoites were injected on day 1 into mice treated with three doses of asparaginase on day 0, 1 and 2. However, asparaginase treatment did not prevent the liver-stage development of PbsWT sporozoites and the asexual-stage parasites could be readily detected in blood from day 6 onwards (Fig. 4j). The data obtained suggested that the extracellular asparagine is essential for the sexual and liver-stage development of PbsaskO parasites. Asparaginase prevents PbsaskO male gametocyte exflagellation. The absence of ookinete formation in mosquitoes fed with PbsaskO parasites from mice treated with asparaginase indicated the possibility of defective gametocytes. To address this, the morphology and numbers of PbsaskO male and female gametocytes formed in mice treated with three doses of asparaginase
Sporozoites in mice treated with asparaginase. Mice were injected intravenously with $10^4$ sporozoites and the appearance of asexual stages was monitored.

Student’s $t$-test.

Asparaginase treatment had no detectable effect on the extracellular asparagine is essential for the development in $\text{Pb}$ ASKO-infected hepatocytes (Fig. 6c,d) and this was in agreement with the absence of asexual stages in $\text{Pb}$ ASKO sporozoite-infected mice treated with asparaginase (Fig. 4j). The absence of liver stage development in $\text{Pb}$ ASKO sporozoite-infected mice treated with asparaginase was also confirmed by performing RT-PCR analysis for total RNA isolated from infected mouse liver samples wherein the product corresponding to parasite GAPDH could not be detected (Supplementary Fig. 5). The results obtained revealed that extracellular asparagine is essential for the in vivo formation of $\text{Pb}$ ASKO EEFs.

Asparaginase prevents $\text{Pb}$ ASKO EEF formation. To analyse the liver-stage development of $\text{Pb}$ ASKO sporozoites with and without asparaginase treatment, immunofluorescence analysis of hepatocytes isolated from mice infected with $10^5$ $\text{Pb}$ ASKO sporozoites using $\text{Plasmodium}$-specific hsp70 antibodies was carried out. For control, hepatocytes isolated from mice infected with $10^5$ $\text{Pb}$ WT sporozoites were used. These studies revealed the existence of exo-erythrocytic forms (EEFs) after 50 h post-infection with $\text{Pb}$ WT- and $\text{Pb}$ ASKO-infected hepatocytes (Fig. 6a). However, the number of EEFs quantified from $\text{Pb}$ ASKO-infected hepatocytes was found to be significantly less when compared with $\text{Pb}$ WT-infected hepatocytes (Fig. 6b). This in turn suggested that the delay observed in the appearance of $\text{Pb}$ ASKO asexual stages on injection of sporozoites in mice (Fig. 3i) was due to the decreased formation of $\text{Pb}$ ASKO EEFs in the liver. Interestingly, EEFs for $\text{Pb}$ ASKO sporozoites were not detected in hepatocytes isolated from $\text{Pb}$ ASKO sporozoite-infected mice treated with asparaginase (Fig. 6c,d) and this was in agreement with the absence of asexual stages in $\text{Pb}$ ASKO sporozoite-infected mice treated with asparaginase (Fig. 4j). The absence of liver stage development in $\text{Pb}$ ASKO sporozoite-infected mice treated with asparaginase was also confirmed by performing RT-PCR analysis for total RNA isolated from infected mouse liver samples wherein the product corresponding to parasite GAPDH could not be detected (Supplementary Fig. 5). The results obtained revealed that extracellular asparagine is essential for the in vivo formation of $\text{Pb}$ ASKO EEFs.

In vivo asparagine injection restores $\text{Pb}$ ASKO exflagellation. Studies were also carried out with asparaginase-treated $\text{Pb}$ ASKO.
values of 30 independent readings. 

Hepatocytes isolated from three different mice infected with 10^5 Pb parasites to assess the effects of in vitro and in vivo asparagine supplementation. To determine whether extracellular asparagine was only required for exflagellation as such or indeed essential for the process of maturation of PbASKO male gametocytes, in vitro exflagellation of PbASKO male gametocytes present in the blood collected from mice treated with asparaginase was examined with different concentrations of asparagine added to the exflagellation medium. For control, PbWT gametocytes treated with asparaginase were used. While a concentration up to a maximum of 5 mg ml\(^{-1}\) did not affect the exflagellation of asparaginase-treated PbWT gametocytes, inhibition was observed at higher concentrations. However, none of the concentrations used were able to restore the exflagellation of PbASKO male gametocytes obtained from mice treated with asparaginase (Fig. 7a). These results suggested that asparagine is essential for the entire process of male gametocyte maturation and its requirement is not just confined to exflagellation.

Since exflagellation of asparaginase-treated PbASKO male gametocytes could not be restored in vitro, asparagine supplementation was attempted in vivo to neutralize the effect of asparaginase by injecting asparaginase-treated mice with six respective doses of 2, 4 and 6 mg asparagine through intraperitoneal route for every 12 h starting from the first dose of asparaginase. Given the limitations associated with the solubility of asparagine and the volume that can be injected intraperitoneally, supplementing mice with concentrations higher than 6 mg per mouse did not seem to be realistic. Although none of the concentrations mentioned above could restore the exflagellation with three doses of asparagine, there was restoration with 6 mg of asparagine when asparaginase treatment was reduced to two doses administered on day 5 and 6 (Fig. 7b; Supplementary Movie 5). The restoration was found to be around 59% in comparison with PbASKO parasites, which did not receive asparagine treatment (Supplementary Fig. 3c). Moreover, the reduction of asparaginase treatment was made because of the high activity of asparaginase used in ALL therapy and even two doses were found to be sufficient for inhibiting PbASKO exflagellation.

To examine whether asparagine supplementation is also required for sexual-stage development in mosquitoes, mosquitoes fed on asparaginase-treated mice in which the PbASKO exflagellation was restored by asparaginase were subjected to neutralize the effect of asparagine supplementation (0.5% w/v) in the feeding solution. While partial restoration observed in PbASKO exflagellation was reflected in the formation of ookinetes, oocysts and sporozoites, no significant differences could be observed with respect to the control mosquitoes that did not receive asparagine supplementation (Fig. 7c–e). The decrease in the formation of ookinetes was found to be accompanied by an increase in the percentage of retorts as well (Fig. 7f,g). Further, PbASKO sporozoites obtained with and without the supplementation of asparagine in mosquitoes showed a similar pattern of liver-stage development...
in mice (Fig. 7h) with a delay in the appearance of asexual stages. All these results suggested that the restoration of exflagellation was adequate for asparagine-treated PbASKO parasites to complete its life cycle. Interestingly, in vivo asparagine supplementation capable of restoring the exflagellation of PbASKO male gametocytes did not support the liver-stage development (Fig. 7i) when mice infected with $10^4$ PbASKO sporozoites were treated with two doses of asparaginase on day 0 and day 1 followed by supplementation with six doses of asparagine (6 mg per mouse; 12 h interval). This in turn suggested that the availability of supplemented asparagine to the exo-erytrocytic stages whose development occurs within the milieu of metabolically active hepatocytes might differ from the gametocyte development in blood stages. While two doses of asparaginase were equally effective in inhibiting the exflagellation (Fig. 7b) and liver-stage development (Fig. 7i) of PbASKO parasites, single dose was found to be insufficient.

**Discussion**

We show here that asparagine is critical for malaria parasite survival, in line with the known remarkable abundance of asparagine in *Plasmodium* proteins. While the presence of asparagine repeats in low complexity regions exists as a hallmark of *P. falciparum* proteins, asparagine serves as one of the most abundant amino acids in proteins of other *Plasmodium* species as well. In comparison with prokaryotic and eukaryotic proteomes with an asparagine frequency of ~4–5% (ref. 36), the frequency of asparagine calculated from the complete coding sequences of *Plasmodium* species was found to fall within a range of 7–14% (P. falciparum ~14%, P. knowlesi ~8%, P. vivax ~7%, P. berghei ~13%, P. yoelii ~13% and P. chabaudi ~12%) with P. falciparum presenting the highest degree of asparagine content. While the functional significance of asparagine repeats in *P. falciparum* still remains unclear, it has been proposed that such asparagine repeats may serve as transfer RNA (tRNA) sponges that can facilitate protein folding by serving as inherent chaperones and control stage-specific expressions by modulating the translational rate. Also, the parasite genome encodes a putative AS despite lacking canonical pathways for amino acid biosynthesis. In the present study, we have characterized the AS of malaria parasites, generated KO parasites in *P. berghei* to address the essentiality of AS in the entire life cycle and determined the importance of extracellular asparagine in the survival of PbASKO parasites by using asparagine treatment to deplete asparagine.

While ammonia-dependent AS-A is known to be present in prokaryotes and in certain protozoan parasites such as *Leishmania* and *Trypanosoma*, AS-B which utilizes glutamine as its preferred amino group donor is found in *E. coli* and higher eukaryotes. Here we use sequence...
comparison together with the enzyme assays performed for recombinant PbAS and total parasite lysates to reveal that the parasite enzyme belongs to the AS-B family. 15, 26–32. The catalytic efficiency of the recombinant parasite enzyme with respect to glutamine \((k_{cat}/K_{m} = 2.7 \times 10^{3} \text{M}^{-1} \text{s}^{-1})\) was found to be at least 14-fold higher in comparison with ammonia \((k_{cat}/K_{m} = 0.19 \times 10^{3} \text{M}^{-1} \text{s}^{-1})\). Gene KO carried out for AS in P. berghei and subsequent comparisons made between the PbASKO phenotype and PbWT for the entire life cycle showed a significant delay in the mortality of mice infected with PbASKO asexual-stage parasites. These results serve as evidence of the synthesis of asparagine, suggesting that asparagine derived from haemoglobin degradation and extracellular sources 17, 26 may not support optimal blood-stage development. More importantly, the sexual stage development of PbASKO parasites was drastically affected, with a nearly 69% reduction in the formation of sporozoites. We also found a significant decline in the formation of PbASKO EEFS, which led to a delay in the appearance of asexual stages and subsequent mortality when PbASKO sporozoites were injected intravenously to initiate liver-stage development in mice. Thus, asparagine synthesis mediated by endogenous AS is crucial for the optimal virulence of malaria parasites especially in the sexual and liver stages where asparagine availabilities in the mosquito host and mouse hepatocytes may prove severely limiting.

Unlike asexual stages wherein haemoglobin degradation serves as an intrinsic source of amino acids, 17, 26 free-living sexual-stage parasites depend primarily on the mosquito haemolymph, and liver-stage parasites must subvert the metabolically active host hepatocytes for extracellular sources 44. Given this context, we were interested to determine whether the absence of endogenous asparagine synthesis is compensated through the utilization of extracellular asparagine and this is in turn responsible for the suboptimal survival of PbASKO sexual and liver stages. To deplete the extracellular asparagine, PbASKO-infected mice were injected with three doses of L-asparaginase (50 IU per mouse), conforming to the ALL treatment regimens in children and adults that involve multiple doses of L-asparaginase (E. coli, Erwinia or pegylated) 22, 23 administered over several days (5,000–10,000 IU m−2 for E. coli asparaginase) 22, 23, 45. Although asparaginase treatment did not have significant effect on PbASKO asexual-stage development per se, sexual-stage development was fully inhibited in mosquitoes, as the PbASKO male gametocytes failed to exflagellate.

While asparagine depletion can affect protein synthesis in general, the expression of a particular subset of proteins related to exflagellation may be hindered severely. In addition, given limitations associated with female gametocyte functionality assessments, abnormalities associated with female gametocytes cannot be ruled out at this stage, and this would require extensive cross-fertilization studies. 46. Interestingly, asparagine content seems to be higher in gametocyte and sporozoite proteins of P. falciparum when compared with the asexual stages. 19. Unlike the asexual stages, which are completed within 24 h for P. berghei followed by the release of merozoites that invade fresh RBCs/reticulocytes, gametocytes reside within the same host cell for several days until gametes form in the mosquito host. 47, 48. Therefore, their amino acid requirements may differ from those of asexual stages, and especially when the host haemoglobin reservoir is depleted during later stages of maturation. Asparagine depletion was found to inhibit the liver-stage development of PbASKO sporozoites in mice, suggesting the key role played by asparagine during the liver stages as well. The absence of EEFS and detectable parasite RNA in hepatocytes isolated from mice infected with PbASKO sporozoites suggests that asparagine depletion may lead to the prevention of PbASKO sporozoite invasion or to an early arrest of liver-stage development. Further investigations are needed to decipher the molecular events underlying the inhibition of sexual- and liver-stage development as well as the significance of these findings with other Plasmodium species, P. vivax in particular.

Our next goal was to examine whether asparagine supplementation can rescue effects of asparaginase treatment on PbASKO parasites. While in vitro supplementation had no effect, exflagellation could be partially restored by reducing asparaginase treatment in mice to 2 days and by supplementing them in vivo with asparagine, suggesting that asparagine is vital for the functional maturation of male gametocytes rather than being required transiently for exflagellation. However, a similar in vivo supplementation failed to restore the liver-stage development of PbASKO sporozoites in asparaginase-treated mice. Considering the development of liver stages in metabolically active hepatocytes and the robust activity of asparaginase utilized in ALL treatment 22, 23, 45, supplemented asparagine may become insufficient and/or less accessible for liver stages. This also denotes that asparagine requirement can serve as an effective target for preventing liver infections.

Altogether our findings lay the foundation for the examination of amino acid requirements in malaria parasites as a versatile therapeutic target for multiple stages (Fig. 8). As malaria parasites are auxotrophic to most of their amino acids, 15, 17, 26, depleting their extracellular sources would effectively interfere with the development of sexual stages in mosquitoes and liver stages in vertebrate hosts. The notion of extracellular amino acid depletion has thus far been pursued only in cancer therapies 22, 23, 49 and the present study highlights its relevance to malaria treatment. For amino acids such as asparagine synthesized in the parasite, this approach may be combined with inhibitors specific to parasite enzymes. It would be interesting to examine whether adenylated sulfoxime derivatives (transition-state analogues capable of inhibiting AS and thus capable of suppressing the proliferation of asparagine-resistant leukemia cell lines 21, 24) may be combined with asparaginase treatment to prevent the sexual- and liver-stage development of PbWT parasites. Furthermore, asparaginase resistance leading to subsequent relapse in patients with ALL has been mainly attributed to the increased expression of AS and to the reduced efflux of asparagine in leukaemia cells 21, 24, that are mediated by various signalling events involving amino acid response, survival-related MEK/ERK and mTORC pathways. 21, 50. However, the existence of a rudimentary amino acid response pathway lacking key homologues of downstream transcriptional

Figure 8 | Targeting amino acid requirements in malaria parasites. Therapeutic options of depleting extracellular sources, targeting biosynthetic enzymes, transporters and AaRS are depicted in red. Brown box highlights the approaches relevant for sexual and liver stages. The approach of targeting AaRS is highlighted in green box. AAs, amino acids; AaRS, aminoacyl-tRNA synthetases.
factors that control the starvation response together with atypical kinase cascades and the absence of TORC and TORC-associated nutrient-sensing mechanisms in Plasmodium suggest that the parasite responses in terms of resistance development may be different from those of cancer cells, and this issue will require further investigation.

Transporters that facilitate the uptake of amino acids in malaria parasites need to be explored for new targets. Interestingly, the physiological relevance of transporters in malaria parasites remains unclear, with only one putative amino acid/auxin permease transporter annotated in the parasite genome. It appears that malaria parasites have evolved with a divergent set of transporters for amino acid uptake and that probable candidates may be those belonging to the major facilitator superfamily and ATP-binding cassette superfamily. Aminoacyl-tRNA synthetases (AARS) of malaria parasites may serve as another set of targets. As AARS inhibitors are known for their anti-bacterial and anti-fungal properties, international efforts have been dedicated to the development of new compounds with better efficacy levels. It has recently been shown that analogues of borrelidin-inhibiting threonyl-tRNA synthetase can offer 100% protection in P. yoelii-infected mice. Inhibitors are also available for asparaginyl-tRNA synthetases, of which tirandamycin B from Streptomyces sp. 17,944 was shown to exhibit in vitro antifilarial activity against Brugia malayi, a parasitic nematode that causes elephantiasis. It would be of interest to examine the antimalarial potential of asparaginyl-tRNA synthetase inhibitors in malaria parasites. Thus, a combination of strategies that deplete extracellular amino acids while targeting biosynthetic enzymes, transporters and AARS in parasites may be explored for the development of a single-exposure radical cure with prophylaxis and chemoprotection. Over the last few years, a collective effort has been made to select compounds with submicromolar IC50 values via high-throughput phenotypic screening tests performed against 25,000 compounds with submicromolar IC50 values via high-throughput phenotypic screening tests performed againstsexual stages of the malaria parasite. Approximately 400 compounds were selected based on their drug- and probe-like properties and were made available through the Open Access compounds were selected based on their drug- and probe-like properties and were made available through the Open Access Reagent Resource Center (MR4), ATCC Manassas Virginia) using TRI reagent for RNA isolation. Total RNA was used and RT-PCR was carried out with RevertAid Reverse Transcriptase (ThermoFisher Scientific) and Phusion High-Fidelity DNA Polymerase (New England Biolabs) using forward (5'-GGCCAGGCTCGTGTTTGATG-3') and reverse (5'-GACGAGGCTTATATGTCACACAAA-3') primers. The restriction sites are underlined. While designing the reverse primer, the stop codon was omitted to ensure the in-frame alignment of PbAS cDNA with the vector-encoded 6xHis-Tag. The PbAS cDNA obtained was then digested with restriction enzymes NotI and XhoI plasmid (Novagen). The construct was then linearized with PstI and SalI. P. berghei ANKA parasites were subjected to digestion followed by digestion with 3'-UTR-specific probe that was synthesized using Klenow Fragment (New England Biolabs) with 5'-32P-dATP. Northern analysis was carried out with 2 μg of RNA and G3PDH reverse primer (5'-GACGAGGCTTATATGTCACACAAA-3'). AATAATTGCAC-3' and PCR was carried out to amplify the 5'-UTR (reverse)-3'-UTR (forward)—5'-GACGAGGCTTATATGTCACACAAA-3'. The amplified 5'- and 3'-UTR products were digested with ApaI and BglII, and Km and NotI, respectively, and cloned into pLP006 plasmid (MRA-70, MRA, ATCC Manassas Virginia). The construct was then linearized with ApaI and NotI, and nucleasefied (Nucleofector 2b, Lonza, Switzerland) into mature schizonts of P. berghei ANKA strain (MRA-311, Malaria Research and Reference Reagent Resource Center (MR4), ATCC Manassas Virginia) using TRI reagent to initiate fresh infections in naive mice. Giemsa-stained thin smears of total RNA prepared from parasitized RBCs/reticulocytes intraperitoneally. Enzyme assays. PbAS activity was measured as described previously using a coupled assay system in which inorganic pyrophosphate generated during asparagine synthesis is quantified by monitoring the oxidation of NADH at 340 nm. Briefly, the assays were carried out in a total volume of 1 ml with different concentrations of aspartate in reaction mixtures containing 50 mM Tris pH 8.0, 10 mM ATP, 10 mM MgCl2, either 5 mM glutamine or 100 mM NH4Cl, 350 μl of pyrophosphate reagent and 3 μg of recombinant PbAS. Radioactive assays were performed in a 25 μl reaction volume containing 20 mM Tris pH 8.0, 20 mM NaCl, 10 mM ATP, 10 mM MgCl2, 5 mM glutamine, 0.5 μg of recombinant PbAS and 30 μl of [15-32P]-dATP (3000 Ci mmol−1). After 1 h, the assays were terminated by heating the reaction mixtures at 95 °C for 15 min followed by centrifugation at 15,000 × g for 1 min. 10 μl aliquots of the supernatants were then spotted on silica gel TLC sheets. The radiolabelled asparagine was separated using a solvent system of isopropanol and water (7:3) and identified by exposing the TLC plates to a phosphorimager screen for 12 h. To detect AS activity in P. berghei parasite lysates, blood collected from infected mice was subjected to saponin and the parasite pellet obtained (~100 mg) was resuspended in 200 μl of 50 mM Tris pH 8.0 containing 50 mM NaCl, 20% glycerol, 0.01% Triton X-100 and 1 mM DTT, and sonicated. The lysate was then centrifuged at 20,000 × g for 2 min to remove the membrane debris and the supernatant obtained was used for assays.

**Methods**

**Over-expression and purification of PbAS.** Total RNA was isolated from P. berghei (PB) ANKA strain (MRA-311, Malaria Research and Reference Reagent Resource Center (MR4), ATCC Manassas Virginia) using TRI reagent (Sigma-Aldrich) according to manufacturer's protocol. For cDNA synthesis, 1 μg of total RNA was used and RT-PCR was carried out with RevertAid Reverse Transcriptase (ThermoFisher Scientific) and Phusion High-Fidelity DNA Polymerase (New England Biolabs) using forward (5'-GGCCAGGCTCGTGTTTGATG-3') and reverse (5'-GACGAGGCTTATATGTCACACAAA-3') primers. The restriction sites are underlined. While designing the reverse primer, the stop codon was omitted to ensure the in-frame alignment of PbAS cDNA with the vector-encoded 6xHis-Tag. In brief, RT reaction was performed at 37 °C for 1 h using reverse primer followed by PCR amplification (40 cycles) at 98 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s using forward and reverse primers. PbAS cDNA obtained was then digested with BamHI and Xhol and cloned into pET-20b (+) plasmid (Novagen). The recombinant plasmid was transformed into E. coli RosettaDE3pLys strain (Novagen) and the cells were grown to an A600 of 1.0 at 30 °C, followed by the induction with 1 mM isopropyl-β-D-thiogalactoside for 12 h at 18 °C. The recombinant PbAS was then purified using Ni2+ -NTA resin (Qiagen). In brief, bacterial cell pellet was resuspended in lysing buffer containing 50 mM Tris pH 8.0, 50 mM NaCl, 20% glycerol, 0.01% Triton X-100 and 1 mM diethionitol with protease inhibitors (aprotinin A and leupeptin) and lysed by sonication. The lysate was centrifuged at 50,000 × g for 1 h and the supernatant was applied onto a column packed with Ni2+ -NTA resin. After washing sequentially with lysing buffer containing 1, 10 and 30 mM imidazole, recombinant PbAS was eluted with lysing buffer containing 150 mM imidazole. The protein was then dialyzed against 0.1 M Tris buffer, quantitated using Bio-Rad Protein Assay Dye Reagent and stored at −80 °C. The total yield of recombinant PbAS was around 0.3–0.4 mg l−1 of bacterial culture with the eluted peak fractions containing protein concentrations of 0.1–0.15 mg ml−1. In-solution trypsin digestion for recombinant PbAS was carried out using mass spectrometry grade Trypsin-ultra (New England Biolabs).
Maintenance of *A. stephensi* mosquitoes. The rearing of *A. stephensi* mosquitoes (*stephensi* sensu stricto, National Institute of Malaria Research, Bangalore) was carried out under standard insectary conditions maintained at 27°C and 75–80% humidity with a 12 h light and dark photo-period as described earlier. Eggs were produced by blood feeding the adult female mosquitoes on Swiss male mice (6–8 weeks old) anaesthetised with ketamine/xylazine. Larvae were reared using standard procedures and the pupae were segregated into cages for adult emergence. The adult mosquitoes were fed with 10% glucose solution containing 0.05% paraminobenzoic acid (feeding solution).

*P. berghei* infection studies in *A. stephensi* mosquitoes. To perform sexual stage studies, 5–7 days old female adult mosquitoes were allowed to feed on Swiss male mice (6–8 weeks old) infected with *P. berghei* WT or ASKO parasites. Mosquito feeding experiments were carried out on day 8 post infection when the blood parasites had already developed with at least two exflagellation centres per field. The fully engorged mosquitoes were then segregated and maintained at 19°C with 75–80% humidity. The mosquito midguts were dissected at 20 h post feeding to remove the blood bolus and quantify the number of ookinetes formed. To determine the number of oocysts, mosquito midguts were dissected on day 10 post feeding and merosome staining was carried out as described earlier. For sporozoite analyses, the salivary glands were dissected on day 10 and the sporozoites were extracted and counted using hemocytometer.

Asparaginase treatment and asparagine supplementation. Asparaginase treatment was carried out in uninfected mice or mice infected with *P. berghei* or *P. falciparum* by injecting 200 μl of a solution of L-asparaginase in saline (Dr Reddy’s Laboratories Ltd., India) through intramuscular route. For exflagellation analyses, the appearance of asexual stages and their subsequent development were monitored by examining the Giemsa-stained blood smears. For liver-stage analyses, mice were treated with three doses of asparaginase on day 5, 6 and 7. For exflagellation analyses, mice were treated with three doses of asparaginase on day 0, 1 and 2 followed by sporozoite injection on day 1. For asparagine supplementation studies, asparaginase treatment was reduced to two doses. Asparagine supplementation in mice was carried out at 12 h interval by injecting 200 μl of asparagine solution (30 mg ml⁻¹ in water) through intraperitoneal route and the restoration of exflagellation was analysed on day 8.

Quantification of plasma asparagine levels. Plasma samples were collected from the per-portal sinus of mice using micro-hematocrit capillary tubes were centrifuged at 2,000 × g for 15 min. 4°C. The supernatant obtained was again centrifuged at 17,000 × g for 5 min to remove any residual cells or cell debris and used immediately for asparagine quantification. In brief, the plasma samples were precipitated with acetone and centrifuged at 15,000 × g for 10 min at 4°C to remove proteins. The supernatant obtained was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and the metabolites were extracted using reversed phase-solid phase extraction cartridges. The eluant was then dried and reconstituted in 0.5% (v/v) acetonitrile followed by 3 h incubation with polyclonal anti-hsp70 rabbit serum (1:50 dilution). After washing with PBS, the hepatocytes were treated for 2 h with FITC-conjugated donkey anti-rabbit secondary antibodies (Santa Cruz; 1:100 dilution). DAPI staining was carried out for 10 min to remove any fluorescent background. After washing with PBS, the hepatocytes were mounted with Fluoroshield (Sigma-Aldrich) were captured using 100x objective. For quantification of EEFs, hepatocytes isolated from three mice fed with 10% glucose solution were used as an internal standard.

Immunofluorescence analyses of EEFs. To analyse the formation of EEFs, mice treated with and without asparagine were infected intravenously with 10⁷ *P. berghei* or *P. falciparum* sporozoites. After 50 h post-infection, hepatocytes from infected mice were isolated as described earlier. The smears were fixed with cold acetone for 10 min at −20°C, blocked subsequently at room temperature for 3 h with PBS containing 3% (w/v) bovine serum albumin followed by 3 h incubation with polyclonal anti-hsp70 rabbit serum (1:50 dilution). After washing with PBS, the hepatocytes were treated for 2 h with FITC-conjugated donkey anti-rabbit secondary antibodies (Santa Cruz; 1:100 dilution). DAPI (1 μg ml⁻¹ in PBS) staining was carried out for 10 min and immunofluorescence images of hepatocytes mounted with Fluoroshield (Sigma-Aldrich) were captured using 100x objective. For quantification of EEFs, hepatocytes isolated from three different mice were pooled and the average number of hepatocytes per field was calculated from 20 different fields to estimate hsp70-positive EEFs present in total number of fields corresponding to 10⁴ hepatocytes and 30 such independent readings were used to plot the graphs.

Exflagellation analyses of male gametocytes. For exflagellation analyses, approximately 5–10 μl of blood collected from tail vein of infected mice was diluted with 1 ml of exflagellation medium and used immediately to count the exflagellation centres as described earlier. To ensure the absence of exflagellation, a minimum of 50 fields were examined over a period of 45 min for the blood collected from each mouse. This was also further confirmed by performing blood-feeding studies in mosquitoes. Live imaging of male gametocyte exflagellation was carried out under bright field with 60 × objective using Leica differential interference contrast microscope. The images were captured at 300 time points for an interval of every 20 ms with 2 × 2 binning. To generate videos, the images were processed using ImageJ software.

Other procedures. Southern, northern and western analyses were carried out using standard protocols. Exflagellation medium was prepared as described earlier. To generate polyvalent serum, mice were immunized with recombinant protein emulsified in Freund’s adjuvant followed by three boosters. IgG purification was carried out using Protein A/G agarose beads.

Statistical analyses. *P* values were determined using unpaired Student’s t-test of Excel software performed for two-tailed distribution and unequal sample variance. *P* < 0.05 was considered as significant. To plot the graphs and perform regression analyses, SigmaPlot software version 10 was used. Error bars given in figures represent the s.d.

Ethics statement. *P. berghei* infection studies in mosquitoes and mice were carried out as per the guidelines of the Committee for the Purpose and Supervision of Experimental animals (CPCSEA), Government of India (Registration No: 48/1999/CPCSEA) and as approved by the Institutional Animal Ethics Committee of the Indian Institute of Science, Bangalore (CAI/ Ethics/319/2013).

References

1. Global Report on Antimalarial Drug Efficacy and Drug Resistance: 2000–2010. World Health Organization, Geneva, Switzerland. Available at http://www.who.int/iris/bitstream/10665/44499/1/9789241500476_eng.pdf
2. Wells, T. N., van Huijsduijnen, R. H. & Van Voorhis, W. C. Malaria: a glass half full? Nat. Rev. Drug Discov. 14, 424–442 (2015).
3. Flannery, E. L., Chatterjee, A. K. & Winderler, E. A. Antimalarial drug discovery - approaches and progress towards new medicines. Nat. Rev. Microbiol. 11, 49–62 (2013).
4. Dondorp, A. M. et al. Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 361, 455–467 (2009).
5. Dondorp, A. M. et al. Artemisinin resistance: current status and scenarios for containment. Nat. Rev. Microbiol. 8, 272–280 (2010).
6. Greenwood, B. Treatment of malaria - a continuing Challenge. N. Engl. J. Med. 371, 474–475 (2014).
7. Tun, K. M. et al. Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect. Dis. 15, 415–421 (2015).
8. Mungthin, M., Bray, P. G., Ridley, R. G. & Ward, S. A. Central role of hemoglobin degradation in mechanisms of action of 4-aminoquinolines, quinoline methanols, and phenanthrene methanols. Antimicrob. Agents Chemother. 42, 2973–2977 (1998).
9. Klonis, N. et al. Artemisinin activity against Plasmodium falciparum requires hemoglobin uptake and digestion. Proc. Natl Acad. Sci. USA 108, 11405–11410 (2011).
10. Coronado, L. M., Nadovich, C. T. & Spadafora, C. Malarial hemozoin: from target to tool. Biochim. Biophys. Acta 1804, 2032–2041 (2014).
11. Burrows, J. N., van Huijsduijnen, R. H., Möhrle, J. J., Oeuvev, C. & Wells, T. N. Designing the next generation of medicines for malaria control and eradication. Malar. J. 12, 187 (2013).
12. Vial, H. et al. CRIMALDDI: platform technologies and novel anti-malarial drug targets. Malar. J. 12, 396 (2013).
13. Wu, G. Functional amino acids in growth, reproduction, and health. Adv. Nutr. 1, 31–37 (2010).
14. Wu, G. Amino acids: metabolism, functions, and nutrition. Amino Acids 37, 1–17 (2009).
15. Gardner, M. J. et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419, 498–511 (2002).
16. Payne, S. H. & Loomis, W. F. Retention and loss of amino acid biosynthetic genes in pathogenic helminths. Int. J. Parasitol. 39, 185–192 (2009).
17. Babbitt, S. E. et al. Plasmodium falciparum44, 11045–11410 (2011).
18. Yadav, M. K. & Swati, D. Comparative genome analysis of six malarial parasites using codon usage bias based tools. Bioinformation 8, 1230–1239 (2012).
19. Filisetti, D. et al. Aminoclaylation of Plasmodium falciparum tRNA(Asn) and insights in the synthesis of asparagine repeats. J. Biol. Chem. 288, 36361–36371 (2013).

20. Dabrowski, A. R. A comparative proteomic analysis of the simple amino acid repeat distributions in Plasmodia reveals lineage specific amino acid selection. PLoS ONE 4, e6231 (2009).

21. Richards, N. G. et al. Studies on mechanism. Trends in Parasitology 4, 81–89 (1988).

22. Tanaka, Y. et al. Suppressive effect of azithromycin on Plasmodium berghei. J. Biol. Chem. 281, 22389–22397 (2006).

23. Singh, G. P. et al. Engineering reduced-immunogenicity enzymes for amino acid depletion responses in mice treated with the anti-cancer agent L-asparaginase. J. Biol. Chem. 284, 32742–32749 (2009).

24. Dunlop, N. S., Finkelstein, A. V. & Galzitskaya, O. V. Trends of amino acid composition of proteins of different taxa. Arch. Biochem. Biophys. 597–608 (2006).

25. Ciustea, M., Gutierrez, J. A., Abbatiello, S. E., Eyler, J. R. & Richards, N. G. Asparagine repeat function in a recombinant human asparagine synthetase. Arch. Biochem. Biophys. 412, 595–605 (2006).

26. Ikeuchi, H. & et al. Analysis of 26 amino acids in human plasma by HPLC using a turbulent flow analysis system. J. Chromatogr. A 1062, 121–128 (2004).

27. Rytting, M. E. Role of L-asparaginase in acute lymphoblastic leukemia: focus on adult patients. Blood Lymphat. Cancer 2, 117–124 2012.

28. Muralidharan, V. & Goldberg, D. E. Asparagine repeats in malaria parasites. J. Mol. Biochem. Parasitol. 13, 277–283 (2003).

29. Galzitskaya, O. V. & Richards, N. G. Asparagine repeat function in the rodent malaria parasite Plasmodium berghei. J. Biol. Chem. 283, 34349–34356 (2008).

30. Janse, C. J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat. Protoc. 1, 143–149 (2006).

31. Cowman, A. F. & Wassom, J. G. A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. J. Cell. Biol. 113, 1033–1042 (1991).

32. Nayar, A. S. et al. Localization and in vivo imaging of malaria parasite liver stages. Trends Parasitol. 16, 536–543 (2000).

33. Shimizu, S., Osada, Y., Kanazawa, T., Tanaka, Y. & Arai, M. Suppressive effect of azithromycin on Plasmodium berghei oocytes in the midgut of mosquitoes. Parasit. Vectors 4, 118 (2011).

34. Tuey, M. G., Warburg, A., Laughinghouse, A., Krettli, A. U. & Miller, L. H. Developmentally regulated infectivity of malaria sporozoites for mosquito salivary glands and the vertebrate host. J. Exp. Med. 175, 1607–1612 (1992).

35. Sharma, G. et al. Analysis of 26 amino acids in human plasma by HPLC using AQC as derivatizing agent and its application in metabolic laboratory. Amino Acids 46, 1253–1263 (2014).

36. Bunpo, P. et al. MR4 staff. in Methods in Anopheles Research (eds Mollison, J. & O’Neill, M.) 141–146 (MR4; ATCC). Insect Pathol. 39, 4821–4833 (2006).
Author contributions
V.A.N. and G.P. conceived and designed the study; V.A.N., D.M., V.S., P.A.S., R.R.P. and M.C.S. performed the experiments; V.A.N., S.K.G. and G.P. analysed the data; and V.A.N. and G.P. wrote the paper.

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