

# Use of a Selective Inhibitor To Define the Chemotherapeutic Potential of the Plasmodial Hexose Transporter in Different Stages of the Parasite's Life Cycle<sup>∇</sup>

Ksenija Slavic,<sup>1†</sup> Michael J. Delves,<sup>2†</sup> Miguel Prudêncio,<sup>3</sup> Arthur M. Talman,<sup>2</sup> Ursula Straschil,<sup>2,4</sup> Elvira T. Derbyshire,<sup>1</sup> Zhengyao Xu,<sup>4</sup> Robert E. Sinden,<sup>2</sup> Maria M. Mota,<sup>3</sup> Christophe Morin,<sup>5</sup> Rita Tewari,<sup>2,4</sup> Sanjeev Krishna,<sup>1</sup> and Henry M. Staines<sup>1\*</sup>

Centre for Infection and Immunity, Division of Clinical Sciences, St. George's, University of London, London SW17 0RE, United Kingdom<sup>1</sup>; Division of Cell and Molecular Biology, Imperial College London, London, United Kingdom<sup>2</sup>; Unidade de Malaria, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal<sup>3</sup>; Institute of Genetics, School of Biology, The University of Nottingham, Nottingham, United Kingdom<sup>4</sup>; and Département de Chimie Moléculaire (UMR 5250, ICMG FR-2607, CNRS), Université Joseph Fourier, Grenoble Cedex, France<sup>5</sup>

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During blood infection, malarial parasites use D-glucose as their main energy source. The *Plasmodium falciparum* hexose transporter (PfHT), which mediates the uptake of D-glucose into parasites, is essential for survival of asexual blood-stage parasites. Recently, genetic studies in the rodent malaria model, *Plasmodium berghei*, found that the orthologous hexose transporter (PbHT) is expressed throughout the parasite's development within the mosquito vector, in addition to being essential during intraerythrocytic development. Here, using a D-glucose-derived specific inhibitor of plasmodial hexose transporters, compound 3361, we have investigated the importance of D-glucose uptake during liver and transmission stages of *P. berghei*. Initially, we confirmed the expression of PbHT during liver stage development, using a green fluorescent protein (GFP) tagging strategy. Compound 3361 inhibited liver-stage parasite development, with a 50% inhibitory concentration (IC<sub>50</sub>) of 11 μM. This process was insensitive to the external D-glucose concentration. In addition, compound 3361 inhibited ookinete development and microgametogenesis, with IC<sub>50</sub>s in the region of 250 μM (the latter in a D-glucose-sensitive manner). Consistent with our findings for the effect of compound 3361 on vector parasite stages, 1 mM compound 3361 demonstrated transmission blocking activity. These data indicate that novel chemotherapeutic interventions that target PfHT may be active against liver and, to a lesser extent, transmission stages, in addition to blood stages.

Up to half a billion people contract malaria each year, and nearly one million die of the disease. The development of resistance to antimalarial drugs is a major obstacle to the treatment of malaria and has made many of the available drugs ineffective. Reports of emerging resistance to artemisinins in Western Cambodia (6, 17–18) warn that the malaria burden may increase, especially if new intervention strategies are not introduced. There is therefore an urgent need to discover new antimalarial drugs that act via novel drug targets. Robust validation of novel targets is critical to this process (30), and for technical and clinical reasons, this has been studied predominantly in the asexual erythrocytic stage of plasmodial parasites. Characterizing the nature of new targets during different stages of the parasite's life cycle can define their essentiality and therefore help in choosing those that are critical to the survival of most stages. Targets that indicate parasites are vulnerable at asexual liver stages of infection that rapidly amplify parasite numbers or that could block transmission have advantages

over those that may make parasites vulnerable at a more limited range of stages of the parasite's life cycle (10).

Predominantly, malarial parasites use glycolysis to generate ATP for their energy requirements, with the more efficient tricarboxylic acid (TCA) cycle being largely disconnected from the energy-generating process, at least during the asexual blood stage (20, 27, 33). Without intracellular energy stores during most of their life cycle, they are dependent on a constant supply of D-glucose from their hosts (reviewed in reference 21). The *Plasmodium falciparum* hexose transporter, PfHT, is the primary D-glucose transporter in *P. falciparum* parasites, enabling the uptake of this essential nutrient across the parasite plasma membrane (34). Using a selective D-glucose derivative (compound 3361) as a competitive inhibitor, PfHT was validated chemically as an antimalarial target (8). Compound 3361 kills asexual blood-stage *P. falciparum* parasites *in vitro*, with a 50% inhibitory concentration (IC<sub>50</sub>) of 16 μM, as well as suppressing the rodent malarial parasite, *Plasmodium berghei*, *in vivo* (8). The importance of PfHT and PbHT (the *P. berghei* orthologue) has been further supported by the demonstration that disruption of the corresponding genes renders asexual blood-stage parasites nonviable (29). Furthermore, using a GFP tag, it was reported that PbHT is expressed throughout the parasite's development inside the

\* Corresponding author. Mailing address: Centre for Infection and Immunity, Division of Clinical Sciences, St. George's, University of London, London SW17 0RE, United Kingdom. Phone: 44(0)2087255572. Fax: 44(0)2087253487. E-mail: hstaines@sgul.ac.uk.

† Joint first authors.

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mosquito vector (29). This suggests PbHT may be functionally important during insect stages.

Here we have investigated additional life cycle stages during which plasmodial hexose transporters might be essential, using compound 3361. The aims of the current investigation were the following: (i) to examine the expression of PbHT during liver-stage development, using the PbHT-GFP-expressing *P. berghei* parasite generated previously (29), (ii) to determine the chemotherapeutic potential of PbHT at the liver stage, using compound 3361 (8), and (iii) with the same compound, to ascertain if targeting of plasmodial hexose transporters can block transmission.

## MATERIALS AND METHODS

**Materials.** Phloretin, bovine serum albumin (BSA), Giemsa stain, dimethyl sulfoxide (DMSO), and Bradford reagent were obtained from Sigma-Aldrich (Dorset, United Kingdom). 3-*O*-(Undec-10-en)-yl-D-glucose (compound 3361) was prepared as described previously (7). [<sup>14</sup>C]-2-Deoxy-D-glucose (2-DOG) was obtained from Amersham (Bucks, United Kingdom). Inhibitors were added to cells as stock solutions in DMSO (the amount of DMSO present during experiments was 0.1% [vol/vol], typically, and DMSO alone was added to all control experiments). In all cases, compound 3361 was added at an intermediate concentration or at the final specified concentration in an appropriate medium for each assay type and left to dissolve for at least 12 h before use.

**Infection of hepatoma cells by sporozoites, expression analysis, and parasite development assays.** Huh-7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 1% (vol/vol) nonessential amino acids, 1% (vol/vol) penicillin-streptomycin, 2 mM L-glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7, and maintained at 37°C with 5% CO<sub>2</sub>.

To obtain parasitized cells for confocal microscopy, Huh-7 cells (5 × 10<sup>4</sup> per well) were seeded in 24-well plates the day before infection. Cells were infected by addition of specific numbers (typically 3 × 10<sup>4</sup>) of *pbht-gfp P. berghei* sporozoites (29), followed by centrifugation at 1,700 × *g* for 7 min. *pbht-gfp P. berghei* sporozoites were obtained by disruption of the salivary glands of freshly dissected infected female *Anopheles stephensi* mosquitoes.

To measure inhibition of intracellular liver-stage parasite development by compound 3361, a firefly luciferase-expressing *P. berghei* line, *PbGFP-Luc<sub>con</sub>*, was used, as described previously (22). Inhibition of parasite development was measured when compound 3361 (with final concentrations ranging from 2 to 100 μM) was added either 1 h before or 2 h after the infection of Huh-7 cells, and infection was measured 44 h after sporozoite addition. The effect of compound 3361 on the viability of Huh-7 cells was assessed by alamarBlue assay (Invitrogen, United Kingdom), using the manufacturer's protocol.

**2-DOG uptake assays with uninfected Huh-7 cells.** Huh-7 cells were seeded at a density of 1 × 10<sup>5</sup> per well of a 24-well plate and allowed to reach near-confluence. Prior to experimentation, cells were washed three times in phosphate-buffered saline (PBS). Experiments were performed at 37°C and initiated by the addition of a 30-μl aliquot of [<sup>14</sup>C]-2-DOG (final concentration of 3.6 μM) to 270 μl of PBS in each well. Uptakes were measured over a 20-min time course in the absence and presence of compound 3361 or phloretin (both at final concentrations of 100 μM). Inhibitors were preincubated with cells for at least 5 min prior to the addition of radiolabel. Uptakes were terminated, and external radiolabel was removed by three rapid washes in 1 ml ice-cold PBS containing 10 mM D-glucose. Cells were lysed in 200 μl 0.1% (vol/vol) Triton-0.1% (wt/vol) SDS in PBS. An aliquot (100 μl) of lysed cells was mixed with 1 ml OptiPhase Supermix scintillant (PerkinElmer, Cambridgeshire, United Kingdom), and the associated radioactivity was measured, using a 1450 MicroBeta Plus scintillation system (Wallac, Turku, Finland). Using the Bradford reagent, the protein concentration in each well was determined for 10 μl of lysed cells diluted 100-fold in distilled water (dH<sub>2</sub>O), using a BSA standard curve.

**Ookinete development assay.** Mice were injected intraperitoneally (i.p.) with 0.2 ml of 6-mg/ml phenylhydrazine (BDH Chemicals Ltd., United Kingdom) (to induce hyperreticulocytosis) 2 to 3 days prior to i.p. inoculation with the *P. berghei* ANKA 2.34 strain. At day four postinfection, parasites were harvested by cardiac puncture of infected mice, and blood was resuspended in ookinete medium (RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine, supplemented with 0.2% (wt/vol) Na<sub>2</sub>CO<sub>3</sub>, 5 U/ml penicillin, 5 μg/ml streptomycin, 50 mg/liter hypoxanthine, 20% (vol/vol) FCS, and 100 μM xanthurenic acid) at

a hematocrit level of 5% before being divided into the wells of a 96-well plate. Compound 3361 and D-glucose were diluted in this medium before being added to appropriate wells at different concentrations either at the point of induction of exflagellation or 2 h later. The parasite cultures were maintained at 19°C for 22 h before being smeared on glass slides and stained with Giemsa stain. Ookinete production was then quantified by visualizing the slides at magnification ×40 under oil immersion. Ookinetes were counted in the field of view and related to the number of erythrocytes present in the same field. Multiple fields were counted at regular intervals in a Saint Andrew's Cross pattern across the whole area of the smear to allow for uneven parasite distribution (13, 28). Ookinete production was calculated as the number of ookinetes present per 1,000 erythrocytes.

**Exflagellation assay.** Mice were infected as described above; on day three postinfection, tail blood was harvested and immediately resuspended in phosphate-buffered saline (PBS), pH 8.0, containing 30 U/ml heparin at a 1:1 (vol/vol) ratio (3). This suspension was added to PBS (pH 8.0) containing different concentrations of compound 3361 and D-glucose. Note that in the absence of additional D-glucose, the only D-glucose present during experimentation is that contained in the blood sample, which is diluted four times, estimated to be between 1.5 and 2 mM. Samples were analyzed by microscopy within 7 min of addition of inhibitor/D-glucose to ensure no exflagellation events were missed. Exflagellation was counted by measuring the number of exflagellation centers in 10 fields under magnification ×20 on a Leica DMR microscope.

**Transmission blocking assay.** Transmission blocking by compound 3361 was assessed using a standard membrane feed assay, as described previously (28). Briefly, blood from 2 infected mice was exsanguinated rapidly and pooled. The infected blood was then mixed with either compound 3361 predissolved in mouse serum or mouse serum containing DMSO only (control) and immediately injected into membrane feeders prewarmed to 39°C. The feeders were then offered to overnight-starved *A. stephensi* (SDA 500 strain) mosquitoes in groups of approximately 100 for 30 min. Unfed mosquitoes were removed the next day, and the remaining mosquitoes were maintained at 19°C and 80% relative humidity on a 12-h light/dark cycle, being fed on a fructose/*p*-aminobenzoic acid solution that was replenished every 2 to 3 days. At 7 days postfeeding, mosquito midguts were dissected and analyzed, using a semiautomated counting macro previously described (5).

**Statistical analysis.** For compound 3361 inhibition assays, a sigmoidal dose-response model with a variable slope was fitted to results, using the GraphPad Prism software program (version 4 for Macintosh). As noted in the text, the effects of compound 3361 were compared, using either an unpaired, two-tailed Student *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest.

**Ethics statement.** All animal work has passed an ethical review process and was approved by the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and in compliance with European Directive 86/609/EEC for the protection of animals used for experimental purposes.

## RESULTS

**Expression of the *P. berghei* hexose transporter during liver-stage development.** We have established that specific inhibition of PfHT by compound 3361 (IC<sub>50</sub> of 16 μM) stops asexual *P. falciparum* growth and multiplication in erythrocytes *in vitro* (8). Here we have analyzed the importance of PbHT for parasite development inside liver cells. Initially, the expression of PbHT was determined during liver-stage development by infecting a hepatoma cell line, Huh-7, with sporozoites bearing a *pbht-gfp* reporter sequence that has replaced the authentic *pbht* sequence in *P. berghei*, as described previously (29). Direct fluorescence imaging of live infected hepatoma cells confirmed that expression of PbHT-GFP was parasite surface localized (being present in the parasite plasma membrane and/or the parasitophorous vacuolar membrane) predominantly at both 24 and 48 h postinfection (Fig. 1A), although some weaker PbHT-GFP signal was also observed within parasites. At 67 h postinvasion, intense PbHT-GFP signal was observed within the parasites, surrounding small clumps of nuclei.

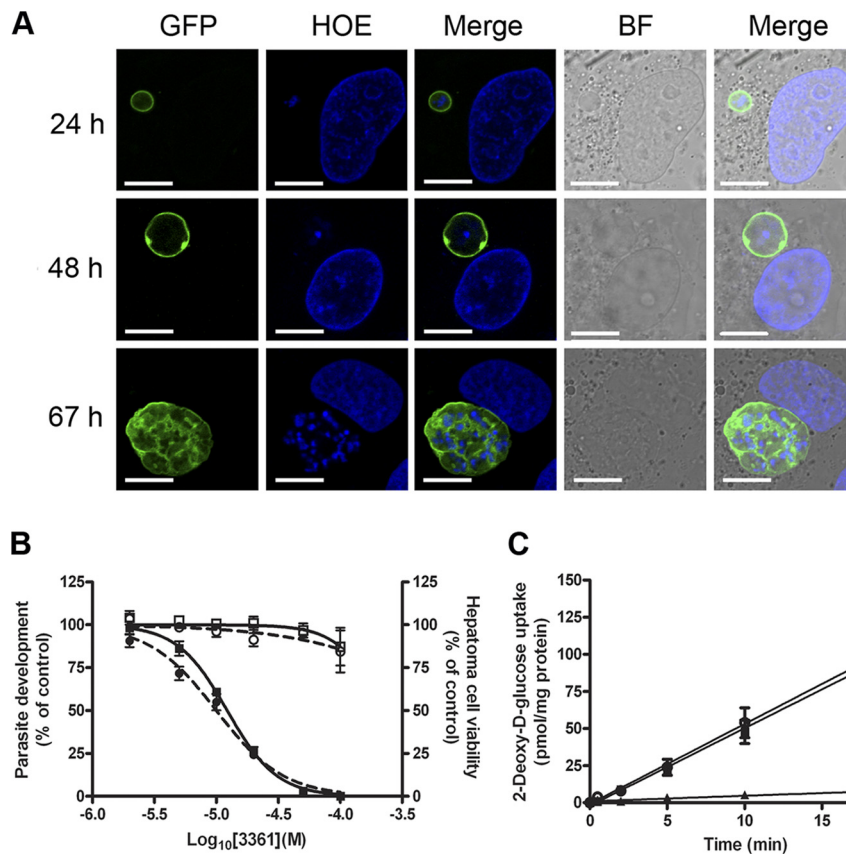


FIG. 1. Expression of GFP-tagged PbHT and the effect of compound 3361 during *P. berghei* development in the human hepatoma cell line Huh-7. (A) Shown are direct fluorescence images of live *pbht-gfp P. berghei*-infected Huh-7 cells at 24, 48, and 67 h postinfection. GFP, *pbht-gfp P. berghei*; HOE, Hoechst 33342, which was used as a nuclear dye; BF, bright-field image. Scale bars = 10  $\mu$ m. (B) The effect of compound 3361 added to Huh-7 cells 1 h preinvasion (squares and solid lines) or 2 h postinvasion (circles and dashed lines) on *P. berghei* development (filled symbols) or Huh-7 cell viability (open symbols), measured at 44 h postinvasion. Infection was assessed by measuring luciferase activity, using parasites (*PbGFP-Luc<sub>con</sub>*) that express firefly luciferase, while Huh-7 cell viability was measured by alamarBlue assay. Data are presented as percentages of luciferase activity or fluorescence intensity (in the case of the alamarBlue assay) measured in paired control experiments performed in the absence of compound 3361 (DMSO present only). Each experiment was performed 3 times in triplicate, and points represent means  $\pm$  SEM. (C) Time courses for the uptake of [<sup>14</sup>C]2-DOG into uninfected Huh-7 cells in the absence (circles) or presence of either compound 3361 (squares) or phloretin (triangles), both at concentrations of 100  $\mu$ M. The extracellular 2-DOG concentration was 3.6  $\mu$ M. Each experiment was performed 3 times in triplicate, and points represent means  $\pm$  SEM.

**Effect of compound 3361 on *P. berghei* liver-stage development.** Since PbHT is expressed in the liver stage of parasite development, we tested the inhibitory properties of compound 3361 (Fig. 1B). *In vitro* development of parasites was determined by measurement of the luminescence of *PbGFP-Luc<sub>con</sub>*-infected hepatoma cells maintained in 96-well plates and incubated with serial dilutions of compound 3361, as described previously (22). Liver cell viability in the presence of compound 3361 was assessed by the alamarBlue assay (see Materials and Methods).

Adding compound 3361 to Huh-7 cells in medium containing approximately 11 mM D-glucose 1 h before initiating sporozoite invasion reduced infection over the next 44 h in a dose-dependent manner, with an IC<sub>50</sub> of 11  $\pm$  2  $\mu$ M (mean  $\pm$  standard error of the mean [SEM];  $n$  = 3). Furthermore, adding compound 3361 to Huh-7 cells 2 h after addition of sporozoites did not significantly change its effect (IC<sub>50</sub> of 12  $\pm$  1 versus 11  $\pm$  2  $\mu$ M;  $P$  = 0.47; unpaired, two-tailed Student's  $t$  test). In this experimental setup, over 95% of parasites that

invade cells do so within 2 h of being added to the cells (23). Therefore, this result shows that the parasite's intracellular development, rather than the invasion of hepatocytes by sporozoites, is affected by compound 3361. Viability of Huh-7 cells was unaffected by concentrations of compound 3361 up to 100  $\mu$ M ( $P$  > 0.05, ANOVA with Tukey's posttest).

In two independent experiments, the effect of D-glucose on the potency of compound 3361 in liver-stage development assays was examined. Development assays were performed in the presence of 11 (as found in RPMI 1640 medium), 21, and 31 mM D-glucose. No differences were observed between the IC<sub>50</sub>s estimated for the effect of compound 3361 on liver-stage parasite development in the presence of each external D-glucose concentration (data not shown).

In an effort to determine if compound 3361 affected the initial steps of D-glucose transport and metabolism in hepatoma cells, its effect on the uptake of D-glucose into uninfected Huh-7 cells was measured (Fig. 1C), using [<sup>14</sup>C]2-DOG (a hexose analogue that can be transported and phosphorylated

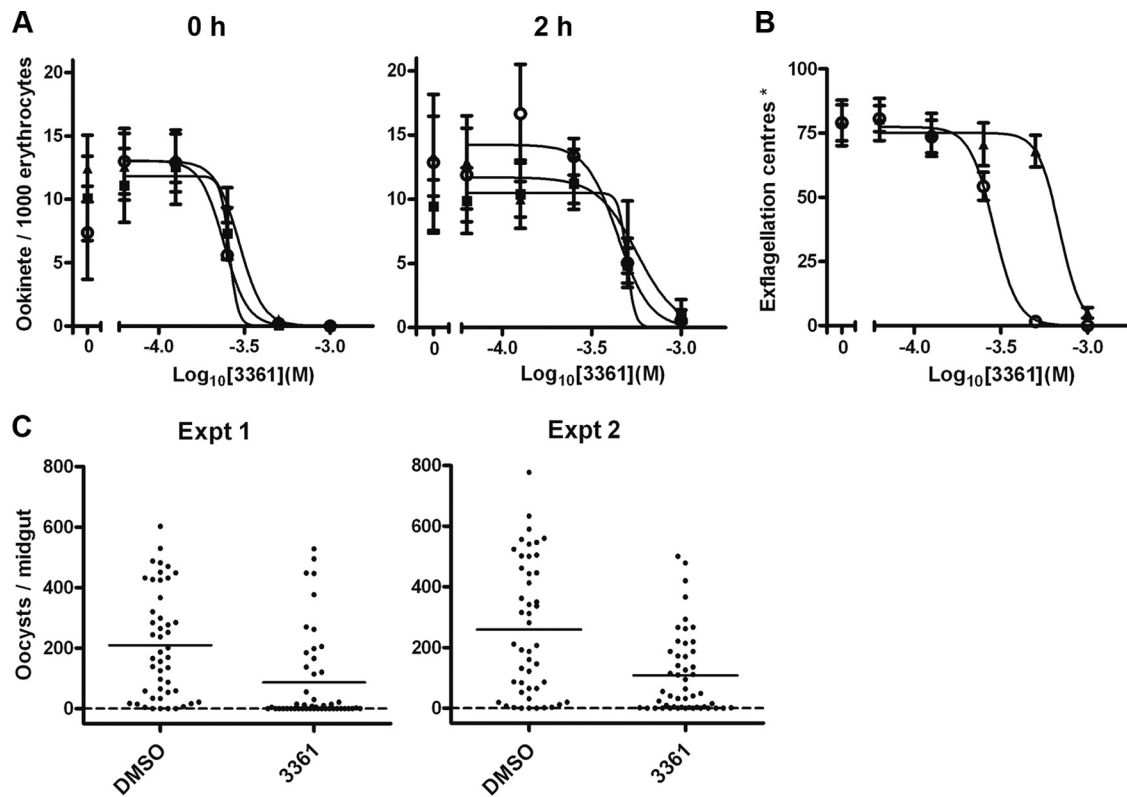


FIG. 2. Effect of compound 3361 on *P. berghei* ookinete development, microgametogenesis, and transmission. (A) Ookinete production was determined after compound 3361 addition at induction of exflagellation (0 h) and 2 h postinduction (2 h) and in the presence of 5.5 (circles), 11 (squares), or 22 (triangles) mM external D-glucose. Data are presented as ookinetes per 1,000 erythrocytes, where points represent means  $\pm$  SEM, and were derived from 3 independent experiments. Control values for addition at induction of exflagellation and at 2 h postinduction are also presented (DMSO present only). (B) Microgametogenesis was determined after compound 3361 addition in the presence of 1.5 to 2 mM (circles) and with an additional 10 mM (triangles) external D-glucose. \*, data are presented as the numbers of exflagellation centers in 10 fields of view (magnification,  $\times 40$ ), where points represent means  $\pm$  SEM, and were derived from 3 independent experiments. Control values are also presented (DMSO present only). (C) Transmission blocking activity of 1 mM compound 3361 added to a mosquito blood feed. Data are presented as the numbers of oocysts in individual mosquitoes fed on blood containing no drug (DMSO present only) or compound 3361 for 2 independent experiments. Solid horizontal lines depict mean oocyst intensities.

but that is not metabolized further). Uptake of [ $^{14}\text{C}$ ]2-DOG (at an external concentration of 3.6  $\mu\text{M}$  and in the complete absence of external D-glucose) into uninfected Huh-7 cells was linear over the 20-min time course used and reached an intracellular level of  $107 \pm 20$  pmol/mg protein (mean  $\pm$  SEM;  $n = 3$ ) by the end. Using a cellular volume of 6  $\mu\text{l}/\text{mg}$  protein, as determined for the human hepatoma cell line HepG2 (1), this equates to a concentration of approximately 18  $\mu\text{M}$ , which is 5-fold higher than the external concentration. The uptake of [ $^{14}\text{C}$ ]2-DOG was not affected significantly by the presence of 100  $\mu\text{M}$  compound 3361 ( $P > 0.05$ , ANOVA with Tukey's posttest) but was inhibited by greater than 90% in the presence of 100  $\mu\text{M}$  D-glucose transport inhibitor, phloretin ( $P < 0.05$ , ANOVA with Tukey's posttest).

**Effect of compound 3361 on *P. berghei* transmission stages.** Previously we have shown that *P. berghei* ookinetes, as well as oocyst- and salivary gland-derived sporozoites, express PbHT-GFP (29). Here we examined the effect of compound 3361 on various developmental stages that take place in the mosquito vector. To test whether D-glucose transport plays a role in malarial parasite sporogonic development, we measured the effect of compound 3361 on ookinete development. Addition

of compound 3361 to parasites in medium containing approximately 11 mM D-glucose at the point of exflagellation induction (Fig. 2A, 0 h) inhibited ookinete development in a dose-dependent manner, with an  $\text{IC}_{50}$  of  $252 \pm 14$   $\mu\text{M}$  (mean  $\pm$  SEM;  $n = 3$ ). The inhibitory effect of compound 3361 on ookinete development was lessened significantly ( $P = 0.0005$ ; unpaired, two-tailed Student's *t* test) when the inhibitor was added 2 h after induction of exflagellation (Fig. 2A, 2 h), with an  $\text{IC}_{50}$  of  $513 \pm 22$   $\mu\text{M}$  (mean  $\pm$  SEM;  $n = 3$ ). The effect of altering the external D-glucose concentration between 5.5, 11, and 22 mM did not produce a significant effect on the estimated  $\text{IC}_{50}$ s for compound 3361 added at either induction of exflagellation or 2 h afterwards ( $P > 0.5$  in both cases; ANOVA with Tukey's posttest).

To further characterize the observed effects of compound 3361 on early sexual development, an exflagellation assay was conducted. Addition of compound 3361 inhibited exflagellation in a dose-dependent manner (Fig. 2B), with an  $\text{IC}_{50}$  of  $286 \pm 12$   $\mu\text{M}$  (mean  $\pm$  SEM;  $n = 3$ ). In this case, the external D-glucose concentration is estimated to be between 1.5 and 2 mM (see Materials and Methods). The presence of an additional 10 mM D-glucose shifted the dose-response curve for the

effect of compound 3361 on exflagellation to the right (2.4-fold shift) and increased the mean  $IC_{50}$  (95% confidence interval [CI];  $n = 3$ ) from 286 (247 to 334) to 689 (518 to 916)  $\mu\text{M}$ , significantly ( $P = 0.001$ ; unpaired, two-tailed Student's  $t$  test).

Having demonstrated that compound 3361 affects parasite development during mosquito stages, we next assessed the ability of compound 3361 to block *P. berghei* transmission (measuring the oocyst burden after gametocyte membrane feeds with parasite-infected blood [5]). In two experiments (Fig. 2C), addition of 1 mM compound 3361 resulted in the mean number of oocysts per mosquito midgut reducing from 210 and 259 in controls to 86 and 109, respectively. Therefore, in both experiments there was an approximately 60% reduction in oocyst intensity. In addition, the infection prevalence (number of mosquitoes with oocysts) was reduced from 91 and 94% in controls to 52 and 78%, respectively, resulting in reductions of 43 and 17%.

## DISCUSSION

In comparison to a range of different drug classes acting against the replicative stages of drug-sensitive parasites in the blood, there are relatively few active against liver and sexual stages (24). Yet effective intervention at the liver stage can cure infection before it causes symptoms, eliminating the risk of progression to severe or fatal disease associated with blood stages, while intervention at the sexual stage can stop transmission. Only the following drugs act against liver stages of infection: pyrimethamine, proguanil, atovaquone, primaquine, and tafenoquine. Currently methodologies limit the application of conventional screening approaches for testing compound libraries on hepatocyte and transmission stages of infection, although new methodologies are under development (5, 22). It is therefore valuable to consider developing drugs against high-biological-value targets that are essential for survival of these stages but which may be less amenable for adaptation to high-throughput screening assays.

D-Glucose uptake mediated by PfHT and PbHT is essential for the survival of erythrocytic stages of *P. falciparum* and *P. berghei* parasites (8, 29). This may be unsurprising, since they need large amounts of D-glucose due to their reliance upon glycolysis for ATP production. The reason for this reliance is not fully understood, but blood-stage asexual parasites have evolved a novel carbon metabolic pathway in which glycolysis is largely disconnected from an intact TCA cycle (a far more efficient ATP production process) that has become branched rather than cyclical (20). Whether this applies to other life cycle stages awaits determination. While these data confirm the importance of D-glucose (and its transport) during blood stages, there are relatively few studies that have investigated energy metabolism in other life cycle stages. The use of a relatively specific inhibitor of the key hexose transporter encoded by *Plasmodium* spp. (PxHT) together with tractable animal and *in vitro* models of infection (such as the recently developed liver-stage infection models [22–23]) has established stages of infection using hexose.

The data presented here demonstrate liver-stage expression of PbHT-GFP, which is consistent with the identification of the *P. yoelii* orthologue of PbHT (PyHT) in a recent proteomic study of liver-stage parasites (32). Data also localize the tagged

hexose transporter to the parasite surface (being present in the parasite plasma membrane and/or the parasitophorous vacuolar membrane) predominantly over the first 48 h of intrahepatic development, in keeping with its localization during other life cycle stages (29, 34). At these time points, the internal PbHT-GFP signal observed might be PbHT in the process of production and trafficking (and/or mislocalized). At 67 h post-invasion, the GFP fluorescence pattern observed here in parasites is similar to that observed in cytomere-stage parasites stained using an antibody against merozoite surface protein 1 (31). This protein is localized to the parasite plasma membrane (rather than the parasitophorous vacuole membrane), which invaginates at the cytomere stage to surround nuclei and leads to merozoite formation. This suggests that PbHT-GFP is also localized to the parasite plasma membrane, although further localization studies with additional reagents would be required to confirm this conclusion definitively.

Compound 3361 not only kills *P. falciparum* parasites in blood culture and *P. berghei* *in vivo* but also inhibits *P. berghei* development in liver stages of infection in a human cell line. The potency of antimalarial activity of compound 3361 for hepatic stages of *P. berghei* (11  $\mu\text{M}$ ) is highly comparable to what we have observed for blood stages of *P. falciparum* infection (16  $\mu\text{M}$ ) (8), consistent with compound 3361 targeting PbHT. Note that these data also compare favorably with the  $K_i$  values for the effect of compound 3361 on D-glucose transport via heterologously expressed PfHT and PyHT, which are 53 and 80  $\mu\text{M}$ , respectively (8–9). The latter hexose transporter shares 96% amino acid sequence identity with PbHT, suggesting that similar results would be obtained for PbHT.

Compound 3361 may alter host cell D-glucose homeostasis (or other unrelated mechanisms) sufficiently to kill the intracellular parasite but not the host cell. However, this is a less likely explanation because compound 3361 did not affect 2-DOG uptake into uninfected Huh-7 cells. Under the conditions used, uptake was linear (extrapolating to the origin) and concentrative (5-fold over 20 min). While it is not possible to rule out completely a faster initial transport step (for example, due to the loss of unphosphorylated [ $^{14}\text{C}$ ]2-DOG during processing), this is consistent with 2-DOG being phosphorylated at the same rate as being transported into the Huh-7 cell cytosol (i.e., transport is rate limiting). These data suggest that compound 3361 does not interfere with either endogenous D-glucose transporters (facilitative glucose transporters, GLUT1 and -2, in Huh-7 cells, predominantly [11]) or kinases (hexokinases II and IV in Huh-7 cells, predominantly [19]). This is consistent with the previous findings that compound 3361 is a weak inhibitor of mammalian GLUT1 and -5 ( $K_i$  values  $> 1$  mM) and has no effect on parasite hexokinase activity at concentrations up to 200  $\mu\text{M}$  (8, 26). Interestingly, the effect of compound 3361 was not sensitive to external D-glucose. However, this may not be surprising if compound 3361 targets the intracellularly localized PbHT and given the role that hepatocytes play in tightly regulating intracellular D-glucose levels as part of their role in systemic glucose homeostasis (2, 4). As is the case with erythrocytes, these data suggest that delivery of inhibitor to the parasite surface (its proposed site of action) is not materially impeded in infected liver cells, consistent with the highly lipophilic nature of the inhibitor.

The data failed to show any effect of compound 3361 on parasite invasion (as opposed to development), since the presence of the inhibitor in the Huh-7 cell culture either 1 h before or 2 h after sporozoite addition made no difference to subsequent development. While this is far from conclusive, it may suggest that the hexose transporter is not essential for sporozoite function. *Plasmodium*-infected mosquitoes are known to upregulate an endogenous D-glucose transporter gene in their salivary glands, suggesting that D-glucose is competed for by salivary gland-localized sporozoites (25). Also, previous studies, including our own PbHT-GFP studies, have demonstrated expression of PbHT in sporozoites (14, 29). However, *P. berghei* sporozoites remain motile, and thus energized, in the absence of D-glucose if one of a number of amino acids is present (15). This suggests that under physiological conditions inhibition of D-glucose uptake by compound 3361 is unlikely to affect sporozoite motility and invasion.

These studies have also explored the dependency of some of the sexual stages of parasite development on the delivery of D-glucose, since our own expression studies (29) and previous proteomic analysis (12; A. M. Talman, J. H. Prietro, S. Marques, M. Lawniczak, R. Frank, A. Ecker, R. S. Stanway, S. Krishna, C. Morin, G. K. Christophides, J. R. Yates III, and R. E. Sinden, unpublished data) have demonstrated the presence of PbHT in transmission stages studied. We have shown here that compound 3361 can inhibit early sexual stages of parasite development. Indeed, ookinete development was hampered pre-fertilization and to a lesser extent post-fertilization. In both cases, there was no clear evidence for competition by excess D-glucose reversing inhibition of ookinete development. Compound 3361 also inhibited exflagellation, and in this case, inhibition was sensitive to the D-glucose concentration. These data suggest that D-glucose transport is essential for the cellular events of microgametogenesis and agree with previous observations that D-glucose maintains the viability (ability to complete microgametogenesis) of male gametocytes in *P. galinaceum* (16). They suggest D-glucose as the key metabolite that powers male gamete motility. The relatively high concentrations of compound 3361 that are needed to inhibit sexual development. These include a reduced requirement for D-glucose, reduced access to the target, and the lack of an appropriate preincubation period with inhibitor or "off-target" effects that kill this stage so that PbHT is not required for survival. With regard to the former, it is worth noting that the concentrations of compound 3361 required to inhibit sexual stages of development are those predicted to maximally block transport of D-glucose via plasmodial hexose transporters (8–9, 26).

In conclusion, here we have demonstrated that PbHT is expressed during intrahepatic parasite development and is localized to the parasite surface. Furthermore, our data are consistent with the hypothesis that targeting plasmodial hexose transporters could be used for causal prophylaxis. We have also demonstrated that transmission-stage parasites are susceptible to a selective inhibitor of plasmodial hexose transporters, although we were only able to demonstrate *P. berghei* transmission blocking activity, using 1 mM compound 3361. Nevertheless, our findings indicate that D-glucose transport

may be a suitable target for mammal-to-mosquito-transmission-blocking drugs.

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