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1 # Use of a selective inhibitor to define the chemotherapeutic potential of
2 the plasmodial hexose transporter in different stages of the parasite's life
3 cycle

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23 **Running title:** Effect of compound 3361 on the *P. berghei* life cycle

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24 **ABSTRACT**

25

26 During blood infection, malarial parasites use D-glucose as their main energy source. The
27 *Plasmodium falciparum* hexose transporter (PfHT), which mediates the uptake of D-glucose into
28 parasites, is essential for survival of asexual blood stage parasites. Recently, genetic studies in the
29 rodent malaria model, *P. berghei*, found that the orthologous hexose transporter (PbHT) is
30 expressed throughout the parasite's development within the mosquito vector, in addition to being
31 essential during intraerythrocytic development. Here, using a D-glucose-derived specific inhibitor
32 of plasmodial hexose transporters, compound 3361, we have investigated the importance of D-
33 glucose uptake during liver and transmission stages of *P. berghei*. Initially, we confirmed the
34 expression of PbHT during liver stage development, using a GFP tagging strategy. Compound
35 3361 inhibited liver stage parasite development, with an IC_{50} value of 11 µM. This process was
36 insensitive to the external D-glucose concentration. In addition, compound 3361 inhibited
37 ookinete development and microgametogenesis, with IC_{50} values in the region of 250 µM (the
38 latter in a D-glucose sensitive manner). Consistent with our findings for the effect of compound
39 3361 on vector parasite stages, 1 mM compound 3361 demonstrated transmission blocking
40 activity. These data indicate that novel chemotherapeutic interventions that target PfHT may be
41 active against liver and, to a lesser extent, transmission stages, in addition to blood stages.

42

43

44 **Key Words:** Glucose; Malaria; Glycolysis; Membrane transport; Compound 3361

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45 **INTRODUCTION**

46

47 Up to half a billion people contract malaria each year and nearly one million die of the disease.
48 The development of resistance to antimalarial drugs is a major obstacle to the treatment of
49 malaria and has made many of the available drugs ineffective. Description of emerging resistance
50 to artemisinins in Western Cambodia (6, 17-18), warns that the malaria burden may increase,
51 especially if new intervention strategies are not introduced. There is, therefore, an urgent need to
52 discover new antimalarial drugs that act via novel drug targets. Robust validation of novel targets
53 is critical to this process (30) and, for technical and clinical reasons, this has been studied
54 predominantly in the asexual erythrocytic stage of plasmodial parasites. Characterising the nature
55 of new targets during different stages of the parasite's life cycle can define their essentiality and
56 therefore help to choose those that are critical to the survival of most stages. Targets that indicate
57 parasites are vulnerable at asexual liver stages of infection that rapidly amplify parasite numbers,
58 or that could block transmission have advantages over those that may make parasites vulnerable
59 at a more limited range of stages of the parasite's life cycle (10).

60

61 Predominantly, malarial parasites use glycolysis to generate ATP for their energy requirements,
62 with the more efficient tricarboxylic acid cycle being largely disconnected from the energy
63 generating process, at least, during the asexual blood stage (20, 27, 33). Without intracellular
64 energy stores during most of their life cycle, they are dependent on a constant supply of D-
65 glucose from their hosts (reviewed in (21)). The *Plasmodium falciparum* hexose transporter,
66 PfHT, is the primary D-glucose transporter in *P. falciparum* parasites, enabling the uptake of this
67 essential nutrient across the parasite plasma membrane (34). Using a selective D-glucose
68 derivative (compound 3361), as a competitive inhibitor, PfHT was validated chemically as an

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69 antimalarial target (8). Compound 3361 kills asexual blood stage *P. falciparum* parasites *in vitro*,
70 with an IC_{50} of 16 μM, as well as suppressing the rodent malarial parasite, *P. berghei*, *in vivo* (8).
71 The importance of PfHT and PbHT (the *P. berghei* orthologue) have been further supported by
72 the demonstration that disruption of the corresponding genes renders asexual blood stage
73 parasites nonviable (29). Furthermore, using a GFP tag, it was reported that PbHT is expressed
74 throughout the parasite's development inside the mosquito vector (29). This suggests PbHT may
75 be functionally important during insect stages.
76
77 Here we have investigated additional life cycle stages during which plasmodial hexose
78 transporters might be essential, using compound 3361. The aims of the current investigation were
79 to i) examine the expression of PbHT during liver stage development, using the PbHT-GFP
80 expressing *P. berghei* parasite generated previously (29), ii) determine the chemotherapeutic
81 potential of PbHT at the liver stage, using compound 3361 (8) and iii) with the same compound,
82 ascertain if targeting plasmodial hexose transporters can block transmission.

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83 **MATERIALS AND METHODS**

84

85 **Materials.** Phloretin, bovine serum albumin (BSA), Giemsa, dimethyl sulphoxide (DMSO) and
86 Bradford reagent were obtained from Sigma-Aldrich (Dorset, UK). 3-*O*-(undec-10-en)-yl-D-
87 glucose (compound 3361) was prepared, as described previously (7). [^{14}C]2-Deoxy-D-glucose (2-
88 DOG) was obtained from Amersham (Bucks, UK). Inhibitors were added to cells as stock
89 solutions in DMSO (the amount of DMSO present during experiments was 0.1% v/v, typically,
90 and DMSO alone was added to all control experiments). In all cases, compound 3361 was added
91 at an intermediate or at the final specified concentration in an appropriate medium for each assay
92 type and left to dissolve for at least 12 h before use.

93

94 **Infection of hepatoma cells by sporozoites, expression analysis and parasite development**
95 **assays.** Huh-7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium
96 supplemented with 10% v/v fetal calf serum (FCS), 1% v/v non-essential amino acids, 1% v/v
97 penicillin/streptomycin, 2 mM L-glutamine and 10 mM 4-(2-hydroxyethyl)-1-
98 piperazineethanesulphonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2.

99

100 To obtain parasitised cells for confocal microscopy, Huh-7 cells (5×10^4 per well) were seeded in
101 24-well plates the day before infection. Cells were infected by addition of specific numbers
102 (typically 3×10^4) of *pbht-gfp P. berghei* sporozoites (29), followed by centrifugation at 1700 g
103 for 7 min. *Pbht-gfp P. berghei* sporozoites were obtained by disruption of the salivary glands of
104 freshly dissected infected female *Anopheles stephensi* mosquitoes.

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106 To measure inhibition of intracellular liver stage parasite development by compound 3361, a
107 firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc$_{con}$, was used, as described previously
108 (22). Inhibition of parasite development was measured when compound 3361 (final
109 concentrations ranging from 2 to 100 µM) was added either 1 h before or 2 h after the infection
110 of Huh-7 cells and infection was measured 44 h after sporozoite addition. The effect of
111 compound 3361 on the viability of Huh-7 cells was assessed by alamarBlue assay (Invitrogen,
112 UK), using the manufacturer's protocol.
113
114 **2-DOG uptake assays in uninfected Huh-7 cells.** Huh-7 cells were seeded at a density of 1 ×
115 10^5 per well of a 24-well plate and allowed to reach near confluency. Prior to experimentation,
116 cells were washed three times in phosphate buffered saline (PBS). Experiments were performed
117 at 37 °C and initiated by the addition of a 30 µl aliquot of [^{14}C]2-DOG (final concentration of 3.6
118 µM) to 270 µl of PBS in each well. Uptakes were measured over a 20 min time course in the
119 absence and presence of compound 3361 or phloretin (both at final concentrations of 100 µM).
120 Inhibitors were preincubated with cells for at least 5 min prior to the addition of radiolabel.
121 Uptakes were terminated and external radiolabel removed by three rapid washes in 1 ml ice-cold
122 PBS containing 10 mM D-glucose. Cells were lysed in 200 µl 0.1% v/v Triton/0.1 % w/v SDS in
123 PBS. An aliquot (100 µl) of lysed cells was mixed with 1 ml OptiPhase Supermix scintillant
124 (PerkinElmer, Cambridgeshire, UK) and the associated radioactivity measured, using a 1450
125 MicroBeta Plus scintillation system (Wallac, Turku, Finland). Using Bradford reagent, the
126 protein concentration in each well was determined for 10 µl of lysed cells diluted 100-fold in
127 dH_2O, using a BSA standard curve.
128

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129 **Ookinete development assay.** Mice were injected intraperitoneally (i.p.) with 0.2 ml of 6 mg/ml
130 phenylhydrazine (BDH Chemicals Ltd, UK) (to induce hyper-reticulocytosis) 2-3 days prior to
131 i.p. inoculation with the *P. berghei* ANKA 2.34 strain. At day 4 post-infection, parasites were
132 harvested by cardiac puncture of infected mice, and blood was resuspended in ookinete medium
133 (RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine, supplemented with 0.2% w/v
134 Na_2CO_3, 5 U/ml penicillin, 5 μg/ml streptomycin, 50 mg/l hypoxanthine, 20% v/v FCS and 100
135 μM xanthurenic acid) at a haematocrit of 5%, before being divided into the wells of a 96-well
136 plate. Compound 3361 and D-glucose were diluted in this medium before being added to
137 appropriate wells at different concentrations either at the point of induction of exflagellation or 2
138 h later. The parasite cultures were maintained at 19 °C for 22 h before being smeared onto glass
139 slides and stained with Giemsa. Ookinete production was then quantified by visualising the slides
140 at 40X magnification under oil immersion. Ookinetes were counted in the field of view and
141 related to the number of erythrocytes present in the same field. Multiple fields were counted at
142 regular intervals in a Saint Andrew's Cross pattern across the whole area of the smear to allow
143 for uneven parasite distribution (13, 28). Ookinete production was calculated as the number of
144 ookinetes present per 1000 erythrocytes and expressed as a percentage of untreated control wells
145 for comparison.
146
147 **Exflagellation assay.** Mice were infected as described above; on day 3 post-infection, tail blood
148 was harvested and immediately resuspended in phosphate-buffered saline (PBS) pH 8.0,
149 containing 30 U/ml of heparin at a 1:1 v/v ratio (3). This suspension was added to PBS (pH 8.0)
150 containing different concentrations of compound 3361 and D-glucose. Note that in the absence of
151 additional D-glucose, the only D-glucose present during experimentation is that contained in the

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152 blood sample, which is diluted 4 times, and is estimated to be between 1.5 and 2 mM. Samples
153 were analysed by microscopy within 7 min of addition of inhibitor/D-glucose to ensure no
154 exflagellation events were missed. Exflagellation was counted by measuring the number of
155 exflagellation centres in 10 fields under 20X magnification on a Leica DMR microscope.
156
157 **Transmission blocking assay.** Transmission blocking by compound 3361 was assessed using a
158 standard membrane feed assay, as described previously (28). Briefly, blood from 2 infected mice
159 was exsanguinated rapidly and pooled. The infected blood was then mixed with either compound
160 3361 predissolved in mouse serum or mouse serum containing DMSO only (control) and
161 immediately injected into membrane feeders prewarmed to 39 °C. The feeders were then offered
162 to overnight-starved *A. stephensi* (SDA 500 strain) mosquitoes in groups of approximately 100
163 for 30 min. Unfed mosquitoes were removed the next day and the remaining mosquitoes
164 maintained at 19 °C and 80% relative humidity on a 12 h light/dark cycle, being fed on a
165 fructose/p-aminobenzoic acid solution that was replenished every 2-3 days. At 7 days post
166 feeding, mosquito midguts were dissected and analysed, using a semi-automated counting macro
167 previously described (5).
168
169 **Statistical analysis.** For compound 3361 inhibition assays, a sigmoidal dose-response model
170 with variable slope was fitted to results, using GraphPad Prism (version 4 for Macintosh). As
171 noted in the text, the affects of compound 3361 were compared, using either an unpaired, two-
172 tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-test.
173
174 **Ethics statement.** All animal work has passed an ethical review process and was approved by the
175 United Kingdom Home Office. Work was carried out in accordance with the United Kingdom

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176 ‘Animals (Scientific Procedures) Act 1986’ and in compliance with ‘European Directive
177 86/609/EEC’ for the protection of animals used for experimental purposes.
178

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179 **RESULTS**

180

181 **Expression of the *P. berghei* hexose transporter during liver stage develpoment.** We have
182 established that specific inhibition of PfHT by compound 3361 (IC_{50} value of 16 μM) stops
183 asexual *P. falciparum* growth and multiplication in erythrocytes *in vitro* (8). Here, we have
184 analysed the importance of PbHT for parasite development inside liver cells. Initially, the
185 expression of PbHT was determined during liver stage development by infecting a hepatoma cell
186 line, Huh-7, with sporozoites bearing a *pbht-gfp* reporter sequence that has replaced the authentic
187 *pbht* sequence in *P. berghei*, as described previously (29). Direct fluorescence imaging of live
188 infected hepatoma cells confirmed expression of PbHT-GFP was parasite surface-localised
189 (being present in the parasite plasma membrane and/or the parasitophorous vacuolar membrane)
190 predominantly at both 24 and 48 h post-infection (Fig. 1A), although some weaker PbHT-GFP
191 signal was also observed within parasites. At 67 h post-invasion, intense PbHT-GFP signal was
192 observed within the parasites, surrounding small clumps of nuclei.

193

194 **Effect of compound 3361 on *P. berghei* liver stage development.** As PbHT (-GFP) is expressed
195 in the liver stage of parasite development, we tested the inhibitory properties of compound 3361
196 (Fig. 1B). *In vitro* development of parasites was determined by measurement of the luminescence
197 of *Pb*GFP-Luc_{con}-infected hepatoma cells maintained in 96-well plates and incubated with serial
198 dilutions of compound 3361, as described previously (22). Liver cell viability in the presence of
199 compound 3361 was assessed by the alamarBlue assay (see Materials and Methods).

200

201 Adding compound 3361 to Huh-7 cells in media containing approximately 11 mM D-glucose 1 h
202 before initiating sporozoite invasion reduced infection over the next 44 h in a dose-dependent

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203 manner, with an IC_{50} value of 11 ± 2 μM (mean ± S.E.M; n = 3). Furthermore, adding compound
204 3361 to Huh-7 cells 2 h after addition of sporozoites did not change significantly its effect (IC_{50}
205 value of 12 ± 1 versus 11± 2 μM; P = 0.47; unpaired, two-tailed, Student's *t*-test). In this
206 experimental setup, over 95% of parasites that invade cells do so within 2 h of being added to the
207 cells (23). Therefore, this result shows that the parasite's intracellular development, rather than
208 the invasion of hepatocytes by sporozoites, is affected by compound 3361. Viability of Huh-7
209 cells was unaffected by concentrations of compound 3361 up to 100 μM (P > 0.05, ANOVA
210 Tukey's post-test).
211
212 In two independent experiments, the effect of D-glucose on the potency of compound 3361 in
213 liver stage development assays was examined. Development assays were performed in the
214 presence of 11 (as found in RPMI 1640 media), 21 and 31 mM D-glucose. No differences were
215 observed between the IC_{50} values estimated for the effect of compound 3361 on liver stage
216 parasite development in the presence of each external D-glucose concentration (data not shown).
217
218 In an effort to determine if compound 3361 affected the initial steps of D-glucose transport and
219 metabolism in hepatoma cells, its effect on the uptake of D-glucose into uninfected Huh-7 cells
220 was measured (Fig 1C), using [^{14}C]2-DOG (a hexose analogue that can be transported and
221 phosphorylated but that is not metabolised further). Uptake of [^{14}C]2-DOG (at an external
222 concentration of 3.6 μM and in the complete absence of external D-glucose) into uninfected Huh-
223 7 cells was linear over the 20 min time course used and reached an intracellular level of 107 ± 20
224 pmol/mg protein (mean ± S.E.M; n = 3) by the end. Using a cellular volume of 6 μl/mg protein,
225 as determined for the human hepatoma cell line, HepG2 (1), this equates to a concentration of

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226 approximately 18 μM, which is 5-fold higher than the external concentration. The uptake of
227 [^{14}C]2-DOG was not affected significantly by the presence of 100 μM compound 3361 ($P > 0.05$,
228 ANOVA with Tukey's post test) but was inhibited by greater than 90% in the presence of 100
229 μM of the D-glucose transport inhibitor, phloretin ($P < 0.05$, ANOVA with Tukey's post-test).
230
231 **Effect of compound 3361 on *P. berghei* transmission stages.** Previously we have shown that *P.*
232 *berghei* ookinetes, as well as oocyst- and salivary gland-derived sporozoites, express PbHT-GFP
233 (29). Here, we examined the effect of compound 3361 on various developmental stages that take
234 place in the mosquito vector. To test whether D-glucose transport plays a role in malarial parasite
235 sporogonic development, we measured the effect of compound 3361 on ookinete development.
236 Addition of compound 3361 to parasites in media containing approximately 11 mM D-glucose at
237 the point of exflagellation induction (Fig 2A, 0 h) inhibited ookinete development in a dose-
238 dependent manner, with an IC_{50} value of 252 ± 14 μM (mean ± S.E.M; n = 3). The inhibitory
239 effect of compound 3361 on ookinete development was lessened significantly ($P = 0.0005$;
240 unpaired, two-tailed, Student's *t*-test) when the inhibitor was added 2 h after induction of
241 exflagellation (Fig 2A, 2 h), with an IC_{50} value of 513 ± 22 μM (mean ± S.E.M; n = 3). The
242 effect of altering the external D-glucose concentration between 5.5, 11 and 22 mM did not
243 produce a significant effect on the estimated IC_{50} values for compound 3361 added at either
244 induction of exflagellation or 2 h afterwards ($P > 0.5$ in both cases; ANOVA with Tukey's post-
245 test).
246
247 To further characterise the observed effects of compound 3361 on early sexual development, an
248 exflagellation assay was conducted. Addition of compound 3361 inhibited exflagellation in a

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249 dose-dependent manner (Fig 2B), with an IC_{50} value of 286 ± 12 μM (mean ± S.E.M; n = 3). In
250 this case, the external D-glucose concentration is estimated to be between 1.5 and 2 mM (see
251 Materials and Methods). The presence of an additional 10 mM D-glucose shifted the dose-
252 response curve for the effect of compound 3361 on exflagellation to the right (2.4-fold shift) and
253 increased the mean IC_{50} value (95% CI; n = 3) from 286 (247 to 334) to 689 (518 to 916) μM,
254 significantly (P = 0.001; unpaired, two-tailed, Student's *t*-test).
255
256 Having demonstrated that compound 3361 affects parasite development during mosquito stages,
257 we next assessed the ability of compound 3361 to block *P. berghei* transmission (measuring
258 oocyst burden after gametocyte membrane feeds with parasite-infected blood (5)). In two
259 experiments (Fig. 2C), addition of 1 mM compound 3361 resulted in the mean number of oocysts
260 per mosquito midgut reducing from 210 and 259 in controls to 86 and 109, respectively.
261 Therefore in both experiments there was an approximate 60% reduction in oocyst intensity. In
262 addition, the infection prevalence (number of mosquitoes with oocysts) was reduced from 91 and
263 94% in controls to 52 and 78%, respectively, resulting in reductions of 43 and 17%.

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264 **DISCUSSION**

265

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

277

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

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288 with tractable animal and *in vitro* models of infection (such as the recently developed liver stage
289 infection models (22-23)) has established stages of infection using hexose.
290

291 The data presented here demonstrate liver stage expression of PbHT-GFP, which is consistent
292 with the identification of the *P. yoelii* orthologue of PbHT in a recent proteomic study of liver
293 stage parasites (32). Data also localise the tagged hexose transporter to the parasite surface (being
294 present in the parasite plasma membrane and/or the parasitophorous vacuolar membrane)
295 predominantly over the first 48 h of intra-hepatic development, in keeping with its localisation
296 during other life cycle stages (29, 34). At these time points, the internal PbHT-GFP signal
297 observed might be PbHT in the process of production and trafficking (and/or mislocalised). At 67
298 h post-invasion, the GFP fluorescence pattern observed here in parasites is similar to that
299 observed in cytomere stage parasites stained using an antibody against merozoite surface protein
300 1 (31). This protein is localised to the parasite plasma membrane (rather than the parasitophorous
301 vacuole membrane), which invaginates at the cytomere stage to surround nuclei and leads to
302 merozoite formation. This suggests that PbHT-GFP is also localised to the parasite plasma
303 membrane, although further localisation studies with additional reagents would be required to
304 confirm this conclusion definitively.
305

306 Compound 3361 not only kills *P. falciparum* parasites in blood culture and *P. berghei in vivo*, but
307 also inhibits *P. berghei* development in liver stages of infection in a human cell line. The potency
308 of antimalarial activity of compound 3361 for hepatic stages of *P. berghei* (11 µM) is highly
309 comparable to what we have observed for blood stages of *P. falciparum* infection (16 µM; (8)),
310 consistent with compound 3361 targeting PbHT. Note that these data also compare favourably
311 with the K_i values for the effect of compound 3361 on D-glucose transport via heterologously

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312 expressed PfHT and PyHT, which are 53 and 80 µM, respectively (8-9). The latter hexose
313 transporter shares 96% amino acid sequence identity with PbHT, suggesting that similar results
314 would be obtained for PbHT.
315
316 Compound 3361 may alter host cell D-glucose homeostasis (or other unrelated mechanisms)
317 sufficiently to kill the intracellular parasite but not the host cell. However, this is a less likely
318 explanation because compound 3361 did not affect 2-DOG uptake into uninfected Huh-7 cells.
319 Under the conditions used, uptake was linear (extrapolating to the origin) and concentrative (5-
320 fold over 20 min). While it is not possible to rule out completely a faster initial transport step (for
321 example due to the loss of unphosphorylated [^{14}C]2-DOG during processing), this is consistent
322 with 2-DOG being phosphorylated at the same rate as being transported into the Huh-7 cell
323 cytosol (*i.e.* transport is rate limiting). These data suggest that compound 3361 does not interfere
324 with either endogenous D-glucose transporters (facilitative glucose transporters, GLUT1 and 2, in
325 Huh-7 cells, predominantly (11)) or kinases (hexokinases II and IV in Huh-7 cells, predominantly
326 (19)). This is consistent with the previous findings that compound 3361 is a weak inhibitor of
327 mammalian GLUT1 and 5 (K_i values > 1 mM) and has no affect on parasite hexokinase activity
328 at concentrations up to 200 µM (8, 26). Interestingly, the effect of compound 3361 was not
329 sensitive to external D-glucose. However, this may not be surprising if compound 3361 targets
330 the intracellularly localised PbHT, and given the role that hepatocytes play in tightly regulating
331 intracellular D-glucose levels, as part of their role in systemic glucose homeostasis (2, 4). As is
332 the case in erythrocytes, these data suggest that delivery of inhibitor to the parasite surface (its
333 proposed site of action) is not materially impeded in infected liver cells, consistent with the
334 highly lipophilic nature of the inhibitor.
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336 The data failed to show any effect of compound 3361 on parasite invasion (as opposed to
337 development), as the presence of the inhibitor in the Huh-7 cell culture either 1 h before or 2 h
338 after sporozoite addition made no difference to subsequent development. While this is far from
339 conclusive, it may suggest that the hexose transporter is not essential for sporozoite function.
340 *Plasmodium*-infected mosquitoes are known to up-regulate an endogenous D-glucose transporter
341 gene in their salivary glands, suggesting that D-glucose is competed for by salivary gland-
342 localised sporozoites (25). Also, previous studies, including our own PbHT-GFP studies have
343 demonstrated expression of PbHT in sporozoites (14, 29). However, *P. berghei* sporozoites
344 remain motile, and thus energised, in the absence of D-glucose, if one of a number of amino acids
345 is present (15). This suggests that in physiological conditions inhibition of D-glucose uptake by
346 compound 3361 is unlikely to affect sporozoite motility and invasion.
347
348 These studies have also explored the dependency of some of the sexual stages of parasite
349 development on the delivery of D-glucose, as our own expression studies (29) and previous
350 proteomic analysis (12); Talman *et al.,* manuscript in preparation) have demonstrated the
351 presence of PbHT in transmission stages studied. We have shown here that compound 3361 can
352 inhibit early sexual stages of parasite development. Indeed, ookinete development was hampered
353 pre-fertilization and to a lesser extent post-fertilization. In both cases, there was no clear evidence
354 for competition by excess D-glucose reversing inhibition of ookinete development. Compound
355 3361 also inhibited exflagellation and, in this case, inhibition was sensitive to the D-glucose
356 concentration. These data suggest that D-glucose transport is essential for the cellular events of
357 microgametogenesis and agree with previous observations that D-glucose maintains the viability
358 (ability to complete microgametogenesis) of male gametocytes in *P. gallinaceum* (16). They
359 suggest D-glucose as the key metabolite that powers male gamete motility. The relatively high

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360 concentrations of compound 3361 needed to inhibit sexual compared with blood and liver stages,
361 may have several explanations. These include a reduced requirement for D-glucose, reduced
362 access to target, the lack of an appropriate pre-incubation period with inhibitor or ‘off-target’
363 effects that kill this stage so that PbHT is not required for survival. With regard to the former, it is
364 worth noting that the concentrations of compound 3361 required to inhibit sexual stages of
365 development are those predicted to maximally block transport of D-glucose via plasmodial
366 hexose transporters (8-9, 26).
367
368 In conclusion, here we have demonstrated that PbHT is expressed during intra-hepatic parasite
369 development and is localised to the parasite surface. Furthermore, our data are consistent with the
370 hypothesis that targeting plasmodial hexose transporters could be used for causal prophylaxis.
371 We have also demonstrated that transmission stage parasites are susceptible to a selective
372 inhibitor of plasmodial hexose transporters, although we were only able to demonstrate *P.*
373 *berghei* transmission blocking activity, using 1 mM compound 3361. Nevertheless, our findings
374 indicate that D-glucose transport may be a suitable target for mammal-to-mosquito transmission
375 blocking drugs.

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377

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496 **Figure Legends**

497

498 FIG.1. Expression of GFP-tagged PbHT and the effect of compound 3361 during *P. berghei*
499 development in the human hepatoma cell line, Huh-7. A. Shown are direct fluorescence images
500 of live *pbht-gfp P. berghei*-infected Huh-7 cells at 24, 48 and 67 h post-infection. GFP, *pbht-gfp*
501 *P. berghei*; HOE, Hoechst 33342, which was used as a nuclear dye; BF, bright-field image. The
502 scale bars equal 10 µm. B. The effect of compound 3361 added to Huh-7 cells 1 h pre-invasion
503 (squares and solid lines) and 2 h post-invasion (circles and dashed lines) on *P. berghei*
504 development (filled symbols) and Huh-7 cell viability (open symbols) measured at 44 h post-
505 invasion. Infection was assessed by measuring luciferase activity, using parasites (*Pb*GFP-Luc_{con})
506 that express firefly luciferase, while Huh-7 cell viability was measured by alamarBlue assay.
507 Data are presented as a percentage of luciferase activity or fluorescence intensity (in the case of
508 the alamarBlue assay) measured in paired control experiments performed in the absence of
509 compound 3361 (DMSO present only). Each experiment was performed 3 times in triplicate and
510 points represent means ± S.E.M. C. Time courses for the uptake of [^{14}C]2-DOG into uninfected
511 Huh-7 cells in the absence (circles) and presence of either compound 3361 (squares) or phloretin
512 (triangles) both at concentrations of 100 µM. The extracellular 2-DOG concentration was 3.6
513 µM. Each experiment was performed 3 times in triplicate and points represent means ± S.E.M.

514

515 FIG. 2. Effect of compound 3361 on *P. berghei* ookinete development, microgametogenesis and
516 transmission. A. Ookinete production was determined after compound 3361 addition at induction
517 of exflagellation (0 h) and 2 h post-induction (2 h) and in the presence of 5.5 (circles), 11
518 (squares) and 22 (triangles) mM external D-glucose. Data are presented as ookinetes per 1000

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519 erythrocytes, where points represent means ± S.E.M., and were derived from 3 independent
520 experiments. Control values for addition at induction of exflagellation and at 2 h post-induction
521 are also presented (DMSO present only). B. Microgametogenesis was determined after
522 compound 3361 addition in the presence of 1.5-2 (circles) and with an additional 10 (triangles)
523 mM external D-glucose. Data are presented as the number of exflagellation centres* in 10 fields
524 of view (40X magnification), where points represent means ± S.E.M., and were derived from 3
525 independent experiments. Control values are also presented (DMSO present only). C. The
526 transmission blocking activity of 1 mM compound 3361 added to a mosquito blood feed. Data
527 are presented as the number of oocysts in individual mosquitoes fed on blood containing no drug
528 (DMSO present only) or compound 3361 for 2 independent experiments. Solid horizontal lines
529 depict mean oocyst intensities.

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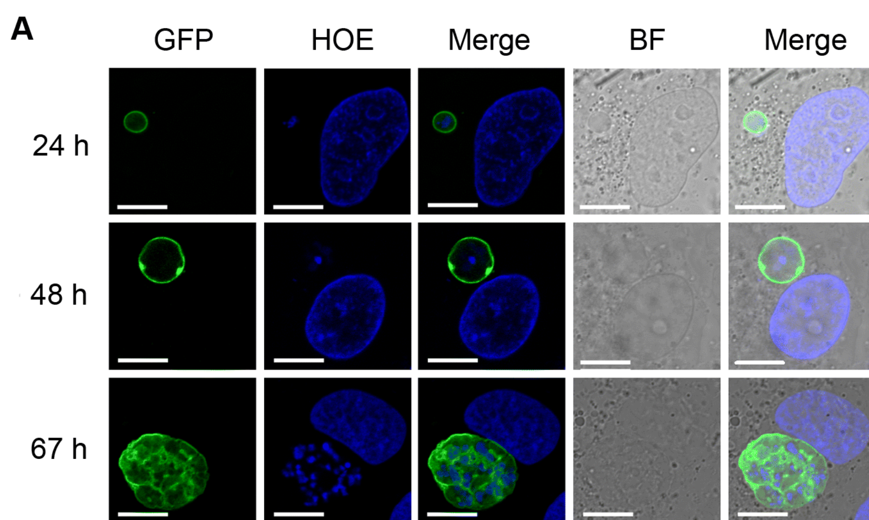

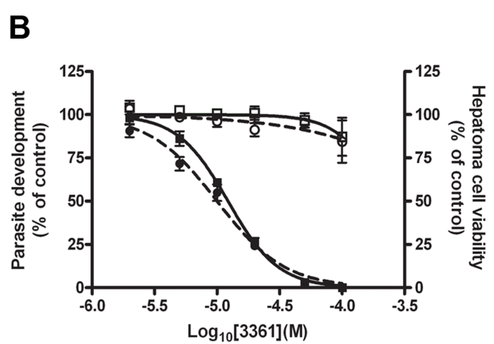

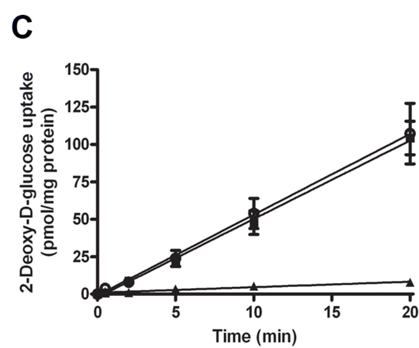

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