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2 **Intermittent preventive treatment with dihydroartemisinin-piperazine in**  
3 **Ugandan schoolchildren selects for *Plasmodium falciparum* transporter**  
4 **polymorphisms that modify drug sensitivity**

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19 Running title: Selection for *P. falciparum* polymorphisms by DP

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21

22 **Abstract**

23 Dihydroartemisinin-piperaquine (DP) offers prolonged protection against malaria, but its  
24 impact on *Plasmodium falciparum* drug sensitivity is uncertain. In a trial of intermittent  
25 preventive treatment in schoolchildren in Tororo, Uganda in 2011-12, monthly DP for one  
26 year decreased the incidence of malaria by 96% compared to placebo; DP once per school  
27 term offered protection primarily during the first month after therapy. To assess the impact  
28 of DP on selection of drug resistance, we compared the prevalence of key polymorphisms in  
29 isolates that emerged at different intervals after treatment with DP. Blood obtained  
30 monthly and at each episode of fever was assessed for *P. falciparum* parasitemia by  
31 microscopy. Samples from 160 symptomatic and 650 asymptomatic episodes of parasitemia  
32 were assessed at 4 loci (N86Y, Y184F, and D1246Y in *pfmdr1* and K76T in *pfcr1*) that  
33 modulate sensitivity to aminoquinoline antimalarials utilizing a ligase detection reaction  
34 fluorescent microsphere assay. For *pfmdr1* N86Y and *pfcr1* K76T, but not the other studied  
35 polymorphisms, the prevalences of mutant genotypes were significantly greater in children  
36 who had received DP within the past 30 days compared to those not treated within 60 days  
37 (86Y 18.0% vs. 8.3%,  $p=0.03$ ; 76T 96.0% vs. 86.1%,  $p=0.05$ ), suggesting selective pressure of  
38 DP. Full sequencing of *pfcr1* in a subset of samples did not identify additional polymorphisms  
39 selected by DP. In summary, parasites that emerged soon after treatment with DP were  
40 more likely than parasites not under drug pressure to harbor *pfmdr1* and *pfcr1*  
41 polymorphisms associated with decreased sensitivity to aminoquinoline antimalarials.

42

43 **Introduction**

44 Malaria, in particular infection with *Plasmodium falciparum*, remains a huge public  
45 health problem, with the highest disease burden in sub-Saharan Africa (1, 2). Important  
46 advances have been made in malaria control recently, with a significant decrease in malaria  
47 burden and progress towards elimination noted in some areas (3). Among key tools in the  
48 control of malaria is intermittent preventive treatment (IPT), the provision of full treatment  
49 courses at regular intervals to high risk populations (4). IPT is standard practice during  
50 pregnancy (IPTp), is recommended in children living in seasonal malaria transmission  
51 settings as seasonal malaria chemoprevention (5), and is being investigated in other  
52 populations (6-9). However, currently IPT is advocated only with sulfadoxine-  
53 pyrimethamine (SP) or a combination of SP and amodiaquine (SP+AQ) (5, 10), regimens  
54 severely compromised by drug resistance in much of Africa (11-13). For malaria treatment,  
55 older regimens have been replaced by artemisinin-based combination therapies (ACTs), and  
56 a similar change may be warranted for IPT.

57 Dihydroartemisinin-piperaquine (DP), which provides rapid killing of most parasites  
58 by dihydroartemisinin, prolonged action against any remaining parasites by piperaquine,  
59 and protection for weeks after therapy due to the long half-life of piperaquine, has recently  
60 been investigated for IPT. Compared to IPTp with SP, IPTp with DP was associated with  
61 lower risks of *P. falciparum* infection and symptomatic malaria during pregnancy in Kenya  
62 (14) and Uganda (15). In Ugandan schoolchildren, monthly IPT with DP was associated with  
63 reduced incidence of malaria and reduced prevalence of parasitemia and anemia compared  
64 to DP given approximately once every three months or placebo (6, 16). Similar results were  
65 observed in Ugandan infants when monthly IPT with DP was compared with daily  
66 trimethoprim-sulfamethoxazole or monthly SP (7). Thus, DP is a promising alternative to SP

67 or SP+AQ for IPT, but its benefits may be undone by the emergence of *P. falciparum*  
68 resistance to either component of the combination.

69 Mediators of decreased drug sensitivity and selective pressures for resistance are  
70 quite well understood for some antimalarial drugs. Resistance to the aminoquinolines  
71 chloroquine and amodiaquine is mediated largely by polymorphisms in putative drug  
72 transporters encoded by *pfcr1* and *pfmdr1* (13, 17), and these polymorphisms are selected in  
73 new infections that emerge soon after therapy with artesunate-AQ (AS/AQ) (18, 19).  
74 Piperaquine is a bisaminoquinoline related to chloroquine and amodiaquine. Resistance to  
75 piperaquine was widely reported during the pre-artemisinin era in China (20), and recently  
76 clinically relevant resistance, with frequent recrudescences after therapy with DP, has been  
77 noted in Cambodia(21-23). However, mechanisms of resistance to piperaquine are  
78 uncertain. Use of DP for treatment (24) or chemoprevention (25) did not select for the  
79 polymorphisms associated with chloroquine resistance in Burkina Faso, but in Uganda  
80 recent treatment with DP selected for *pfmdr1* mutations associated with decreased  
81 sensitivity to aminoquinolines (26). Interestingly, some other antimalarials, notably  
82 lumefantrine, which is a component of the Ugandan first-line antimalarial regimen  
83 artemether-lumefantrine (AL), exert the opposite selective pressure. Thus, new infections  
84 emerging within two months of treatment with AL showed selection of wild-type sequences  
85 at the *pfcr1* K76T and *pfmdr1* N86Y and D1246Y alleles (26-29); mutant sequences are  
86 selected at these same alleles by aminoquinolines. Of recent concern has been resistance to  
87 artemisinins, manifest as delayed parasite clearance after therapy, in Southeast Asia (22, 30-  
88 32), but recent studies utilizing clinical, parasitological, and molecular markers (33, 34)  
89 suggest that the artemisinin-resistant phenotype is not yet prevalent in Uganda (26, 35, 36)  
90 or other parts of Africa (37, 38).

91 Taken together, available evidence suggests that DP may select for the same *P.*  
92 *falciparum* polymorphisms as other aminoquinolines, leading to decreased treatment or  
93 preventive efficacy of DP, but data on the effects of IPT with DP are very limited. We  
94 therefore assessed the prevalences of key polymorphisms in isolates that emerged at  
95 different intervals after treatment with DP using samples from a recent trial evaluating IPT  
96 with DP in Ugandan schoolchildren.

97

## 98 **Methods**

99 **Clinical trial.** Study samples were from a randomized, double-blinded, placebo-  
100 controlled trial conducted in Tororo, Uganda from 2011 to 2012 (6, 39). In brief, 740  
101 schoolchildren aged 6–14 years from one primary school in Mulanda sub-county, Tororo  
102 District were enrolled and randomized 1:1:1 to one of three study arms: DP monthly, DP  
103 once per school term (four treatments over 12 months), or placebo. DP was administered  
104 according to weight based guidelines and treatment was directly observed. Finger-prick  
105 blood samples were obtained at enrollment, every month, and with every episode of fever  
106 to assess for malaria infection by thick blood smear, and for storage on filter paper.  
107 Episodes of uncomplicated malaria were treated with AL. Children were followed for 12  
108 months. The trial was approved by the Uganda National Council for Science and Technology  
109 and the Makerere University School of Medicine Research and Ethics Committee and  
110 registered at ClinicalTrials.gov (NCT01231880). Molecular studies were also approved by the  
111 University of California, San Francisco Committee on Human Research.

112 **Selection of samples for testing of parasite polymorphisms.** We considered all  
113 samples that were positive for *P. falciparum* parasitemia based on evaluation of Giemsa-  
114 stained thick blood smears, as previously described (6). A total of 160 symptomatic and

115 1,522 asymptomatic episodes of *P. falciparum* parasitemia were documented. The number  
116 of samples analysed was determined by estimating the power for two-sample comparison  
117 of proportions using effect sizes observed for each mutant polymorphism in a recent study  
118 in Tororo (0.34 for *pfmdr1* N86Y, 0.11 for *pfmdr1* D1246Y, 0.04 for *pfmdr1* 184F, and 0.09  
119 for *pfcr1* K76), fixing  $\alpha$  at 0.05 (26). The sample size giving the maximum power was  
120 considered in the analysis. From these estimates, we analysed all 160 samples from  
121 symptomatic episodes, all 50 samples from children with recurrent parasitemia within 13-30  
122 days of prior therapy with DP, and 600 samples randomly selected from children with either  
123 recurrent parasitemia >30 days after prior therapy with DP or from the control arm of the  
124 study. All samples were analyzed for 4 common *P. falciparum* polymorphisms known to be  
125 associated with drug sensitivity: *pfcr1* K76T, and *pfmdr1* N86Y, Y184F, and D1246Y. A subset  
126 of 25 samples from children with prior DP therapy within 13-30 days and 25 randomly  
127 selected paired samples from children in the control arm (each pair matched for collection  
128 within 15 days of each other) were subjected to sequencing of the complete *pfcr1* gene.

129 **Characterization of 4 *pfcr1* and *pfmdr1* polymorphisms.** DNA was extracted from  
130 filter paper blood spots into 100  $\mu$ L of water using Chelex-100 as previously described (40).  
131 Gene fragments spanning all loci of interest were amplified in nested reactions (26), and  
132 failed reactions were repeated. To detect polymorphisms, multiplex ligase detection  
133 reaction–fluorescent microsphere assays were performed as previously described (26, 41).

134 **Sequencing of *pfcr1*.** For a subset of samples *pfcr1* was sequenced from DNA samples  
135 as previously described (42) with minor modifications. Briefly, *pfcr1* was amplified in 3  
136 nested-PCR reactions, covering exons 1-2, 3-8, and 9-13, using the published primer  
137 sequences. For both rounds of PCR, each 25  $\mu$ L reaction contained 2 mM MgSO<sub>4</sub>, 200  $\mu$ M  
138 each dNTP, 1  $\mu$ M each primer, 1X PCR Buffer, and 2U Platinum Taq DNA Polymerase High

139 Fidelity (Invitrogen). Conditions for all reactions were 94oC for 2 min; 30 cycles of 94oC for  
140 20 sec, 47oC for 10 sec, and 60oC for 3 min; and a final extension at 60oC for 5 min.  
141 Amplicons were cloned with the TOPO-TA Cloning Kit for Sequencing and transfected into  
142 One Shot TOP10 chemically competent *E. coli* (Invitrogen) according to the manufacturer's  
143 instructions. Colonies were grown overnight under kanamycin selection, picked, and  
144 incubated in LB broth with kanamycin. Plasmid DNA was purified using the PureLink Quick  
145 Plasmid Miniprep Kit (Invitrogen), digested with *EcoRI* to confirm the insert size, and then  
146 sequenced (Eurofins) using M13 forward and reverse primers. DNA sequence data were  
147 assembled and edited, and mutations were detected by alignment and comparison it to the  
148 expected sequence using CodonCode Aligner v. 5.1.5. Multiple clones were sequenced to  
149 distinguish true polymorphisms from PCR errors, including at least 3 clones for all but 3  
150 fragments, for which 2 clones were sequenced.

151 **Statistical analysis.** Data analysis was done using Stata version 14 (StataCorp).  
152 Outcomes of interest were the prevalence of pure mutant alleles for each locus of interest.  
153 The exposure variable of interest was duration since prior DP dose, evaluated as a  
154 categorical variable split into 13 – 30, 31 – 60, and > 60 days (including the no treatment  
155 control group) since the last treatment. Associations between outcomes and duration since  
156 last treatment and differences between prevalences of *pfcr*t alleles were measured using  
157 Fisher's exact test and expressed as relative risk. In all analyses, a 2-tailed P value <0.05 was  
158 considered statistically significant.

159

## 160 **Results**

161 **Study samples.** A total of 740 schoolchildren aged 6 – 14 years were randomized to  
162 one of the 3 study arms in the parent study and followed for one year from 2011 to 2012. As

163 previously reported, compared to either DP once per school term (approximately every 3  
164 months) or placebo, monthly DP offered strong protective efficacy against malaria (6). For  
165 this sub-study, samples collected from children with blood smears positive for *P. falciparum*  
166 were analyzed (Table 1). As expected due to the protective efficacy of monthly DP, fewer  
167 samples were available from this study arm than from children who received placebo or DP  
168 once per school term. A total of 810 samples from 160 symptomatic and 650 asymptomatic  
169 episodes of parasitemia were assessed (Table 1). Samples were analysed for common  
170 polymorphisms in *pfmdr1* and *pfcr1*. Genotyping results were available for *pfcr1* K76T in 806  
171 (99.5%) samples and for *pfmdr1* N86Y, N184Y, and D1246Y in 800 (98.8%), 810 (100%), and  
172 784 (96.8%) samples, respectively, and these results were included in the analysis.

173 **Prevalence of *pfcr1* and *pfmdr1* polymorphisms.** The prevalence of the 4 studied  
174 polymorphisms was similar to that in contemporaneous samples from Tororo that were  
175 reported previously (43). For two polymorphisms, *pfcr1* K76T and *pfmdr1* N86Y, the  
176 prevalence of mutant genotypes was significantly higher in samples from children who had  
177 received DP within 30 days compared to those from children who had not received DP  
178 within 60 days (Table 2). For the other studied polymorphisms the prevalence of genotypes  
179 did not differ between children who had or had not received recent therapy with DP.  
180 Matching for duration since a prior episode, there was no difference in the prevalence of  
181 *pfcr1* and *pfmdr1* mutant alleles between samples from children with symptomatic or  
182 asymptomatic parasitemia (data not shown).

183 **Sequencing of *pfcr1*.** As DP may select for additional polymorphisms in *pfcr1*, we  
184 sequenced the gene in a subset of 25 parasitemic samples under strong selective pressure  
185 as indicated by emergence within 30 days of prior therapy with DP and in 25 paired samples  
186 collected near the same date from children who did not receive DP. We successfully



187 sequenced the full gene in 17 pairs. We identified 9 polymorphisms, 6 of which are  
188 commonly reported in African isolates (Supplemental Table 1). All isolates had the *pfcr*t 72-  
189 76 CVIET or a mix of the CVIET and CVMNT haplotype, except for one isolate that had the  
190 *pfcr*t 72S mutation, resulting in the SVIET haplotype (in all 6 clones from a patient not  
191 receiving DP). Two additional polymorphisms, L50P and F112I, were each identified in at  
192 least 2 clones from a single isolate, the 50P mutation in a control isolate and the 112I  
193 mutation in an isolate from a child recently treated with DP (Supplemental Table 2). We  
194 found 9 *pfcr*t haplotypes; the majority (76% in the DP arm and 65% in the control arm) were  
195 mutant at the six loci that are commonly mutant in Africa (74I, 75E, 76T, 220S, 271E, 371I)  
196 (17). Overall, we saw no evidence that DP selected for novel *pfcr*t polymorphisms in  
197 Ugandan children.

198

#### 199 Discussion

200 Monthly IPT with DP was highly efficacious in reducing the risks of symptomatic  
201 malaria, parasitemia, and anemia in Ugandan schoolchildren (6). However, the  
202 chemoprophylactic benefits of a long-acting antimalarial such as piperazine may be  
203 accompanied by selection of drug resistant parasites (13). We tested whether DP selected  
204 for parasites with genotypes associated with altered sensitivity to aminoquinolines.  
205 Compared to parasites not under drug pressure, those that emerged within 30 days of IPT  
206 with DP were more likely to harbor two mutations, *pfmdr*1 86Y and *pfcr*t 76T; these  
207 mutations are associated with resistance to chloroquine and amodiaquine (36, 43-45).  
208 Thus, the marked preventive efficacy of IPT with DP may be accompanied by selection of  
209 decreased sensitivity to aminoquinolines.

210 Resistance to chloroquine and amodiaquine is mediated primarily by polymorphisms  
211 in putative drug transporters encoded by *pfcr1* and *pfmdr1* (13, 46). The *pfcr1* 76T and  
212 *pfmdr1* 86Y and 1246Y mutations are selected in new infections that emerge soon after  
213 therapy with regimens including chloroquine or amodiaquine (47). Piperaquine is a related  
214 bisaminoquinoline, but mechanisms of resistance are uncertain, and studies of the selective  
215 pressure exerted by DP have yielded conflicting results. Specifically, use of DP for treatment  
216 (48), or chemoprevention (25), did not select for the polymorphisms associated with  
217 aminoquinoline resistance in Burkina Faso, but, in Uganda, recent treatment with DP  
218 selected for the *pfmdr1* 86Y and 1246Y mutations (26). Our new results shed additional  
219 light on this area. In the setting of IPT in schoolchildren, recent receipt of DP was associated  
220 with selection of the *pfmdr1* 86Y and *pfcr1* 76T mutations, but not the *pfmdr1* 1246Y  
221 mutation. Differing results may have been due to the changing baseline of polymorphism  
222 prevalence in Uganda, with decreasing prevalence of *pfmdr1* 1246Y and *pfcr1* 76T over time.  
223 Differences in results between West and East Africa may also be explained by differences in  
224 parasite backgrounds; of note, the *pfmdr1* 1246Y mutation, which until recently was  
225 widespread in Uganda, has consistently been uncommon in Burkina Faso (24, 25, 28).

226 Importantly, although we lack a head-to-head comparison, it appears that DP does  
227 not select as readily as other ACTs for key transporter mutations. In multiple studies the  
228 selective pressure of AS/AQ was marked (49), including a recent trial that showed the  
229 prevalence of the pure *pfmdr1* 86Y mutation to rise from 59% at baseline to 99% in  
230 recurrent infections within one month of treatment (50). AL also exerts strong selective  
231 pressure, but in the opposite direction, with selection of wild type *pfcr1* K76 and *pfmdr1* N86  
232 and N1246 sequences in parasites that emerge soon after therapy (19, 29). Our recent  
233 findings indicate that DP selects for resistance in a manner similar to that of the other

234 aminoquinolines, but associations between recent therapy and transporter polymorphisms  
235 were less marked, suggesting that the selective pressure of DP is lower than that of other  
236 regimens. This difference might be due to different mechanisms of transport for  
237 piperazine, a much larger molecule compared to chloroquine or amodiaquine.

238         We were concerned that IPT with DP might select for additional resistance-mediating  
239 *P. falciparum* polymorphisms. Polymorphisms in addition to those commonly described in  
240 African isolates have been identified in other regions, in some cases with biochemical and  
241 clinical consequences (51, 52). Sequencing of *pfcr* in a subset of samples either under or  
242 not under the selective pressure of DP identified a few previously unidentified *pfcr*  
243 mutations, but it did not suggest that additional polymorphisms were selected by DP.

244         Our results have important implications for the use of DP for IPT. Although it offers  
245 great promise for decreasing the malaria burden, DP use may be accompanied by selection  
246 of parasites with decreased sensitivity to DP, and also to the related ACT AS/AQ.  
247 Consideration of the opposite resistance pressures of different antimalarials has led some to  
248 recommend multiple or rotating first-line antimalarial regimens (53). For example, AS/AQ  
249 and AL have opposite selective pressures on *pfcr* and *pfmdr1* such that each regimen  
250 should blunt selection of resistance to the other. Our results are consistent with a prior  
251 study in Uganda indicating that DP has similar selective pressure to that of AS/AQ. Thus,  
252 considering resistance selection, using DP in IPT might be best advised when the standard  
253 treatment regimen is AL, such that the treatment and IPT regimens offer mutual protection  
254 against selection of resistance. Further, our results suggest that, with changing treatment  
255 and control practices, continued surveillance for clinical, biochemical, and molecular  
256 markers of antimalarial drug resistance in Africa is an important priority.

257

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268

269 **Potential conflicts of interest**

270 All authors report no conflicts of interest.

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497 **Table 1. Characteristics of study children that supplied samples and of episodes selected**  
 498 **for analysis**

<b>Characteristics of children with at least one episode of parasitemia</b>	<b>N=389</b>
Median age (IQR)	9 (7 – 11)
Median duration of observation in days (IQR)	366 (365 – 368)
Female sex (n, %)	209 (53.7)
Study group n (%)	
Placebo	178 (45.8)
IPT once a school term	178 (45.8)
Monthly IPT	33 (8.4)
<b>Characteristics of episodes of parasitemia</b>	<b>N=810</b>
Malaria classification n (%)	
Asymptomatic episodes	650 (80.2)
Clinical episodes	160 (19.8)
Study group n (%)	
Placebo	334 (41.3)
IPT once a school term	419 (51.7)
Monthly IPT	57 (7.0)
Duration since prior treatment n (%)	
15 – 30 days	50 (6.2)
31 – 60 days	122 (15.1)
61 – 90 days	170 (20.9)
>90 days	134 (16.5)
No treatment	334 (41.2)

499

500 Table 2: Prevalence of *P. falciparum* pure mutant alleles stratified by time since last dose of DP.

Allele	Days since last dose of DP	Prevalence of wild-type, mixed, and mutant alleles n/N (%)			RR for mutant genotype (95% CI)	p-value
		Wild type	Mixed	Mutant		
<i>pfmdr1</i> N86Y	>60 <sup>a</sup>	189/630 (30.0)	389/630 (62.7)	52/630 (8.3)	1	
	31 – 60	53/120 (44.2)	57/120 (47.5)	10/120 (8.3)	1.01 (0.53 – 1.93)	0.98
	13 – 30	25/50 (50.0)	32/50 (32.0)	9/50 (18.0)	2.18 (1.14 – 4.16)	0.03
<i>pfmdr1</i> N184Y	>60 <sup>a</sup>	143/638 (22.4)	458/638 (68.8)	37/638 (5.8)	1	
	31 – 60	25/122 (20.5)	84/122 (68.8)	13/122 (10.7)	1.84 (1.01 – 3.35)	0.07
	13 – 30	21/50 (42.0)	28/50 (56.0)	1/50 (2.0)	0.34 (0.05 – 2.46)	0.51
<i>pfmdr1</i> D1246Y	>60 <sup>a</sup>	261/616 (42.4)	292/616 (47.4)	63/616 (10.2)	1	
	31 – 60	59/120 (49.2)	51/120 (42.5)	10/120 (8.3)	0.81 (0.43 – 1.54)	0.62
	13 – 30	24/48 (50.0)	21/48 (43.7)	3/48 (6.3)	0.61 (0.20 – 1.87)	0.61
<i>pfcr1</i> K76T	>60 <sup>a</sup>	9/635 (1.4)	79/635 (12.4)	547/635 (86.1)	1	
	31 – 60	1/121 (0.8)	13/121 (10.7)	107/121 (88.4)	1.03 (0.96 – 1.10)	0.56
	13 – 30	1/50 (2.0)	1/50 (2.0)	48/50 (96.0)	1.11 (1.04 – 1.19)	0.05

501 <sup>a</sup>Includes those given no drug (placebo group)