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

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

Abstract

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

Introduction

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

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

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

Methods

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

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

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

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

Sequencing of *pfcrt*. For a subset of samples *pfcrt* was sequenced from DNA samples as previously described (42) with minor modifications. Briefly, *pfcrt* was amplified in 3 nested-PCR reactions, covering exons 1-2, 3-8, and 9-13, using the published primer sequences. For both rounds of PCR, each 25 µL reaction contained 2 mM MgSO4, 200 µM each dNTP, 1 µM each primer, 1X PCR Buffer, and 2U Platinum Taq DNA Polymerase High

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

Statistical analysis. Data analysis was done using Stata version 14 (StataCorp). Outcomes of interest were the prevalence of pure mutant alleles for each locus of interest. The exposure variable of interest was duration since prior DP dose, evaluated as a categorical variable split into 13 – 30, 31 – 60, and > 60 days (including the no treatment control group) since the last treatment. Associations between outcomes and duration since last treatment and differences between prevalences of *pfcrt* alleles were measured using Fisher's exact test and expressed as relative risk. In all analyses, a 2-tailed P value <0.05 was considered statistically significant.

Results

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

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

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

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

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

Discussion

Monthly IPT with DP was highly efficacious in reducing the risks of symptomatic malaria, parasitemia, and anemia in Ugandan schoolchildren (6). However, the chemoprophylactic benefits of a long-acting antimalarial such as piperaquine may be accompanied by selection of drug resistant parasites (13). We tested whether DP selected for parasites with genotypes associated with altered sensitivity to aminoquinolines. Compared to parasites not under drug pressure, those that emerged within 30 days of IPT with DP were more likely to harbor two mutations, *pfmdr1* 86Y and *pfcrt* 76T; these mutations are associated with resistance to chloroquine and amodiaquine (36, 43-45). Thus, the marked preventive efficacy of IPT with DP may be accompanied by selection of decreased sensitivity to aminoquinolines.

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

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

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

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

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

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Potential conflicts of interest

All authors report no conflicts of interest.

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

Characteristics of children with at least one episode of parasitemia	**N=389**
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)
Characteristics of episodes of parasitemia	**N=810**
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)

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		
pfmdr1 N86Y	>60[a]	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
pfmdr1 N184Y	>60[a]	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
pfmdr1 D1246Y	>60[a]	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
pfcrt K76T	>60[a]	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

[a]Includes those given no drug (placebo group)

Supplemental Table 1. Non-synonymous polymorphisms detected by sequencing of *pfcrt* in Ugandan isolates.

pfcrt Allele	Treatment Arm[a]	Wild type N (%)	Mixed N (%)	Mutant N (%)	P-value[b]
L50P	DP	17 (100)	0 (0)	0 (0)	p = 1.000
	Control	16 (94)	1 (6)	0 (0)	
C72S	DP	17 (100)	0 (0)	0 (0)	p = 1.000
	Control	16 (94)	0 (0)	1 (6)	
M74I	DP	0 (0)	2 (12)	15 (88)	p = 0.6552
	Control	0 (0)	4 (24)	13 (76)	
N75E	DP	0 (0)	2 (12)	15 (88)	p = 0.6552
	Control	0 (0)	4 (24)	13 (76)	
K76T	DP	0 (0)	2 (12)	15 (88)	p = 0.6552
	Control	0 (0)	4 (24)	13 (76)	
F112I	DP	16 (94)	1 (6)	0 (0)	p = 1.000
	Control	17 (100)	0 (0)	0 (0)	
A220S	DP	0 (0)	2 (12)	15 (88)	p = 1.000
	Control	1 (6)	0 (0)	16 (94)	
Q271E	DP	0 (0)	2 (12)	15 (88)	p = 1.000
	Control	1 (6)	0 (0)	16 (94)	
R371I	DP	1 (6)	0 (0)	16 (94)	p = 0.60
	Control	3 (18)	0 (0)	14 (82)	

[a]Samples from the DP arm were parasites emerging 15-30 days after therapy with DP; controls were from the placebo group that did not receive DP.

[b]P-values are based on comparison of prevalence between treatment arms using Fisher's exact test.

Supplemental Table 2. *Pfcrt* haplotypes seen in sequenced samples.

Haplotype	Treatment arm		L50P	C72S	M74I	N75E	K76T	F112I	A220S	Q271E	R371I
	DP N (%)	Control N (%)									
1	13 (76)	11 (65)	L	C	I	E	T	F	S	E	I
2	1 (6)	2 (12)	L	C	M/I	N/E	K/T	F	S	E	R
3	0 (0)	1 (6)	L	C	M/I	N/E	K/T	F	S	E	I
4	0 (0)	1 (6)	L	S	I	E	T	F	S	E	I
5	0 (0)	1 (6)	L	C	I	E	T	F	A	Q	R
6	1 (6)	0 (0)	L	C	I	E	T	F	A/S	Q/E	I
7	1 (6)	0 (0)	L	C	M/I	N/E	K/T	F	A/S	Q/E	I
8	1 (6)	0 (0)	L	C	I	E	T	F/I	S	E	I
9	0 (0)	1 (6)	L/P	C	I	E	T	F	S	E	I

Loci with two alleles indicate a mixed genotype.