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MECHANISMS OF RESISTANCE

Associations between Malaria-Preventive Regimens and *Plasmodium falciparum* Drug Resistance-Mediating Polymorphisms in Ugandan Pregnant Women

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ABSTRACT Intermittent preventive treatment in pregnancy (IPTp) with monthly sulfadoxine-pyrimethamine (SP) is recommended for malaria-endemic parts of Africa, but efficacy is compromised by resistance, and, in recent trials, dihydroartemisinin-piperaquine (DP) has shown better antimalarial protective efficacy. We utilized blood samples from a recent trial to evaluate selection by IPTp with DP or SP of *Plasmodium falciparum* genetic polymorphisms that alter susceptibility to these drugs. The prevalence of known genetic polymorphisms associated with altered drug susceptibility was determined in parasitemic samples, including 375 collected before IPTp drugs were administered, 125 randomly selected from those receiving SP, and 80 from those receiving DP. For women receiving DP, the prevalence of mixed/mutant sequences was greater in samples collected during IPTp than that in samples collected prior to the intervention for PfMDR1 N86Y (20.3% versus 3.9%; $P < 0.001$), PfMDR1 Y184F (73.0% versus 53.0%; $P < 0.001$), and PfCRT K76T (46.4% versus 24.0%; $P < 0.001$). Considering SP, prior to IPTp, the prevalence of all 5 common antifolate mutations was over 92%, and this prevalence increased following exposure to SP, although none of these changes were statistically significant. For two additional mutations associated with high-level SP resistance, the prevalence of PfDHFR 164L (13.7% versus 4.0%; $P = 0.004$), but not PfDHPS 581G (1.9% versus 3.0%; $P = 0.74$), was greater in samples collected during IPTp compared to those collected before the intervention. Use of IPTp in Uganda selected for parasites with mutations associated with decreased susceptibility to IPTp regimens. Thus, a potential drawback of IPTp is selection of parasites with decreased drug susceptibility.

KEYWORDS malaria, *Plasmodium falciparum*, intermittent preventive therapy, dihydroartemisinin-piperaquine, sulfadoxine-pyrimethamine

Chemoprevention is recommended to prevent malaria in high-risk groups (1). Among chemoprevention strategies, the World Health Organization recommends intermittent preventive treatment of malaria in pregnancy (IPTp) with monthly sulfadoxine-pyrimethamine (SP) in areas of Africa with moderate to high malaria transmission (2). IPTp with SP has offered modest protective efficacy against malaria, but this efficacy has been threatened by resistance to both components of this regimen (3, 4).

The components of SP target two folate pathway enzymes, dihydrofolate reductase (PfDHFR) and dihydropteroate synthetase (PfDHPS), offering synergistic activity against *Plasmodium falciparum* (5). A number of well-characterized mutations decrease the

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activities of pyrimethamine and sulfadoxine against PfDHFR and PfDHPS, respectively. Five mutations (PfDHFR 51I, 59R, and 108N and PfDHPS 437G and 540E) have been very common in *P. falciparum* in Uganda and surrounding countries for many years, although 540E is uncommon in West Africa (6, 7). These mutations mediate an intermediate level of resistance to SP (5). Additional mutations, notably PfDHFR 164L and PfDHPS 581G and 613T, that mediate high-level resistance to SP have been seen in other regions. These additional mutations have been uncommon in *P. falciparum* from most of Africa, but studies have shown increasing prevalence in some areas, including PfDHFR 164L in southwestern Uganda (8–10) and PfDHPS 581G in parts of Tanzania (11), western Uganda (9, 10), and eastern Democratic Republic of Congo (12). In women receiving IPTp with SP, the PfDHPS 581G mutation was associated with decreased malarial preventive efficacy (13) and decreased birth weight (14) in Malawi, and the prevalence of this mutation was greater in parasites collected at delivery than those collected before the initiation of IPTp in Ghana (15). It is of interest to determine if, in Uganda, the use of monthly SP for IPTp selects for an increased prevalence of mutations that mediate resistance to this regimen.

In light of concerns regarding limited efficacy of IPTp with SP in prior studies (2) and worsening *P. falciparum* resistance to SP, there has been interest in alternative regimens for IPTp. Recent clinical trials in Uganda (16, 17) and Kenya (18) demonstrated that IPTp with the artemisinin-based combination therapy (ACT) dihydroartemisinin-piperaquine (DP) has superior preventive efficacy against malaria during pregnancy and against placental malaria compared to SP. When used for chemoprevention, DP benefits from the long (3- to 4-week) half-life of piperaquine, offering strong preventive efficacy with monthly dosing in children (19, 20) and pregnant women (16–18). However, the poor pharmacokinetic match between dihydroartemisinin and piperaquine engenders a risk of selection of parasites with decreased susceptibility to piperaquine. In Southeast Asia, resistance to both dihydroartemisinin and piperaquine has led to frequent failures after treatment of malaria with DP (21, 22). Importantly, the known genetic markers associated with piperaquine resistance in Southeast Asia, increased copy number of plasmepsin genes (23, 24) and novel mutations in PfCRT (25) are not prevalent in Africa (4). However, the antimalarial activity of piperaquine may also be impacted, albeit less markedly, by polymorphisms that are common in Uganda. Piperaquine is a bisquinoline related to the aminoquinolines chloroquine and amodiaquine. Mutations associated with aminoquinoline resistance, in particular, PfMDR1 86Y and PfCRT 76T, have been associated with decreased piperaquine susceptibility and selected by prior treatment with DP in some studies, although selection does not appear to be as marked as that with other aminoquinolines (26–28), and the prevalence of these mutations has decreased over time in Uganda (9, 10).

To consider selection by IPTp with DP or SP of potential drug resistance mediators in the context of changing parasite genotypes in Uganda over time, we compared the prevalence of key *P. falciparum* resistance markers known to be circulating in Uganda between parasites collected from pregnant women before or after the initiation of IPTp.

RESULTS

Study samples. Study subjects were pregnant women in Busia District, Uganda, who were enrolled between 16 and 20 weeks of gestation in a randomized trial comparing monthly SP or DP as IPTp (17). Study subjects were all HIV uninfected and at least 16 years of age. To reach our desired sample size, we randomly selected *P. falciparum*-positive samples with parasitemia of at least 10 parasites/μl, both from samples collected prior to administration of the first dose of IPTp and from samples from the SP arm following the administration of IPTp with SP. We selected all samples from the DP arm following the administration of IPTp with DP with parasitemia of at least 1 parasite/μl, as this arm had fewer positives due to the greater preventive efficacy of DP (Fig. 1).

Selection of transporter polymorphisms. We compared the prevalence of 4 genetic polymorphisms that have been common in Africa in *P. falciparum* collected

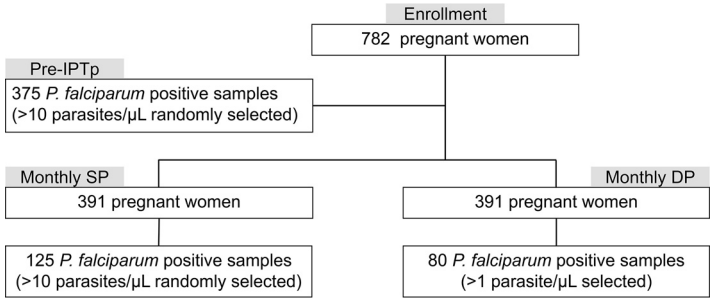

FIG 1 Sample selection. Means of selecting samples are shown.

from women before the onset of IPTp or during the course of monthly IPTp with DP. For PfMDR1 86Y, PfMDR1 184F, and PfCRT 76T, mutations were more prevalent in parasites collected while women received IPTp with DP than in parasites collected before the onset of IPTp or in parasites collected while women received IPTp with SP (Table 1). For the other tested polymorphism, PfMDR1 D1246Y, the prevalence of the mutation was similar in parasites collected before or during IPTp. IPTp with SP was not associated with changes in transporter polymorphisms.

Selection of antifolate polymorphisms. We compared the prevalence of antifolate polymorphisms known to be prevalent in Africa in *P. falciparum* collected from women before the onset of IPTp or during the course of monthly IPTp with SP. For all 5 mutations that have been common in *P. falciparum* circulating in East Africa for many years (PfDHFR 51I, PfDHFR 59R, PfDHFR 108N, PfDHPS 437G, and PfDHPS 540E), prevalence was >92% in parasites collected before IPTp, and mutation prevalence was slightly but not significantly higher in parasites collected during IPTp with SP (Table 2). One of these mutations, PfDHPS 540E was less common in parasites from women receiving IPTp with DP than in those collected before therapy or from women receiving IPTp with SP. One additional mutation (PfDHFR 164L) associated with higher-level resistance to SP was more common in parasites collected during IPTp with SP than those collected before therapy.

DISCUSSION

We studied associations between the use of two regimens for IPTp and the selection of *P. falciparum* genetic polymorphisms associated with decreased parasite susceptibility to those regimens. SP, the established regimen for IPTp across Africa, is chal-

TABLE 1 Associations between exposure to IPTp and transporter gene polymorphisms

Locus	Drug exposure	Mutation prevalence (no. mixed and mutant/total no. samples [%])	Prevalence ratio[a] (95% CI)	*P* value	Prevalence ratio[b] (95% CI)	*P* value
pfmdr1N86Y	Before IPTp	12/307 (3.9)	Reference			
	SP	7/113 (6.2)	1.58 (0.64–3.93)	0.32	Reference	
	DP	15/74 (20.3)	5.19 (2.53–10.6)	<0.001	3.27 (1.40–7.64)	0.005
pfmdr1Y184F	Before IPTp	158/298 (53.0)	Reference			
	SP	52/116 (44.8)	0.85 (0.67–1.06)	0.15	Reference	
	DP	54/74 (73.0)	1.38 (1.16–1.64)	<0.001	1.63 (1.27–2.08)	0.0002
pfmdr1D1246Y	Before IPTp	22/299 (7.4)	Reference			
	SP	8/109 (7.3)	0.99 (0.46–2.18)	0.99	Reference	
	DP	5/74 (6.8)	0.92 (0.36–2.35)	0.78	0.92 (0.31–2.70)	0.88
pfcrtK76T	Before IPTp	72/300 (24.0)	Reference			
	SP	25/96 (26.0)	1.09 (0.73–1.61)	0.68	Reference	
	DP	26/56 (46.4)	1.93 (1.37–2.74)	<0.001	1.78 (1.15–2.77)	0.01

[a]Reference group is Before IPTp.
[b]Reference group is SP.

TABLE 2 Associations between exposure to IPTp and antifolate gene polymorphisms

Locus	Drug exposure	Mutation prevalence (no. mixed and mutant/total no. samples [%])	Prevalence ratio[a] (95% CI)	*P* value	Prevalence ratio[b] (95% CI)	*P* value
pfdhfrN51I	Before IPTp	266/281 (94.7)	Reference			
	DP	66/70 (94.3)	1.00 (0.93–1.06)	0.99	Reference	
	SP	108/113 (95.6)	1.01 (0.96–1.06)	0.80	1.01 (0.95–1.09)	0.73
pfdhfrC59R	Before IPTp	282/305 (92.5)	Reference			
	DP	66/70 (94.3)	1.02 (0.95–1.09)	0.80	Reference	
	SP	116/121 (95.9)	1.04 (0.99–1.09)	0.28	1.02 (0.95–1.09)	0.73
pfdhfrS108N	Before IPTp	306/310 (98.7)	Reference			
	DP	67/67 (100)	1.01 (1.00–1.02)	0.99	Reference	
	SP	119/119 (100)	1.01 (1.00–1.03)	0.58	NA	NA
pfdhfrI164L	Before IPTp	9/223 (4.0)	Reference			
	DP	6/66 (9.1)	2.25 (0.83–6.10)	0.12	Reference	
	SP	14/102 (13.7)	3.40 (1.52–7.60)	0.004	1.51 (0.61–3.73)	0.47
pfdhpsA437G	Before IPTp	277/281 (98.6)	Reference			
	DP	61/61 (100.0)	1.01 (1.00–1.03)	0.99	Reference	
	SP	91/93 (97.8)	0.99 (0.96–1.03)	0.64	0.98 (0.95–1.01)	0.52
pfdhpsK540E	Before IPTp	302/309 (97.7)	Reference			
	DP	39/58 (67.2)	0.69 (0.57–0.82)	<0.001	Reference	
	SP	110/112 (98.2)	1.00 (0.98–1.04)	0.99	1.46 (1.22–1.75)	<0.001
pfdhpsA581G	Before IPTp	9/303 (3.0)	Reference			
	DP	0/51 (0.0)	NA[c]	0.37	Reference	
	SP	2/106 (1.9)	0.64 (0.14–2.89)	0.74	NA	0.99
pfdhpsA613S	Before IPTp	0/295 (0.0)	Reference			
	DP	0/68 (0.0)	NA	NA	Reference	
	SP	0/111 (0.0)	NA	NA	NA	NA

[a]Reference group is Before IPTp.
[b]Reference group is SP.
[c]NA, not applicable, as the prevalence ratio cannot be calculated.

lenged by resistance, and parasites from women who received IPTp with SP had increased prevalence of one resistance-associated parasite mutation, PfDHFR 164L. Use of DP, an artemisinin-aminoquinoline combination under study as a new agent for IPTp, was associated with significant increases in the prevalence of mutations associated with decreased aminoquinoline susceptibility.

SP has been the standard agent for IPTp for many years. It benefits from ease of dosing (a single monthly oral dose) and established safety, but its efficacy has been limited for many years by a high prevalence of mutations in the target enzymes PfDHFR and PfDHPS that mediate resistance to its component drugs (3, 4). In this setting, the primary resistance concern is the selection of additional mutations that mediate high-level SP resistance. The use of SP was associated with the selection of one of these mutations, PfDHFR 164L. The lack of selection of 581G may have been due to the low numbers of parasites with these mutations circulating in eastern Uganda, limiting opportunities for selection. The results offer some reassurance that the use of SP will not rapidly select for parasites with high-level SP resistance. However, the prevalence of these mutations has recently been increasing in western Uganda (9, 10), suggesting that continued use of SP, in addition to the use of antifolates as antibacterials and for prophylaxis in HIV infection, may be selecting for *P. falciparum* highly resistant to SP.

DP is a promising alternative to SP for IPTp, offering much improved antimalarial preventive efficacy, although a change in policy to adopt DP for IPTp has been slow due to concerns about selection of parasites resistant to ACTs, potential drug-related congenital malformations or toxicity, and the unexpected result that SP offers similar protection against poor birth outcomes as does DP, possibly due to nonmalarial

activities of SP (16–18). The antimalarial activity of DP may be altered by mutations in the drug transporters PfCRT and PfMDR1 that are common in Africa and mediate resistance to the related aminoquinolines chloroquine and amodiaquine, although results have been inconsistent (4). Use of DP for the treatment (29, 30) or prevention (27, 28, 31) of malaria has been associated with the selection of transporter mutations in some, but not other, studies. Considering this background, it is of interest that, in our study, use of DP was strongly associated with the selection of the two transporter mutations most clearly associated with aminoquinoline resistance, PfCRT 76T and PfMDR1 86Y, and with another mutation, PfMDR1 184F, that may stabilize the fitness of parasites with the other mutations. Thus, the use of DP in this setting selected for parasites with reduced susceptibility to amodiaquine, a component of the widely used ACT artesunate-amodiaquine, and possibly reduced susceptibility to piperaquine. Interestingly, parasites with these mutations are more sensitive than wild-type parasites to lumefantrine, a component of artemether-lumefantrine, the ACT that is the first-line antimalarial in Uganda and much of Africa.

In Southeast Asia, DP efficacy is severely challenged by resistance to both components of this regimen, associated with additional polymorphisms, specifically, kelch protein propeller domain mutations for artemisinins and plasmepsin gene amplification and/or novel PfCRT mutations for piperaquine (23–25). Due to cost constraints, we did not test for these polymorphisms in this study, but multiple other recent studies have shown their near absence in Uganda and other sites in Africa (4).

Our study had some limitations. First, due to the high protective efficacy of DP, this arm of our study had few available samples for study. However, despite a low sample size, significant associations between DP use and parasite mutations were seen. Second, due to a low number of available samples, we studied all samples in the DP arm, compared to a random set of samples, with a higher parasitemia cutoff, in the pretreatment and SP arms. The higher parasite densities in the pretreatment and SP-treated groups might have enhanced genotyping success and so affected comparisons, especially considering identification of minority genotypes. Third, a fair percentage of our assays were unsuccessful, probably due to the relatively low parasitemias present in many of our samples, which were mostly associated with asymptomatic parasitemia. Fourth, as noted above, due to cost constraints, we did not characterize the full genomes of study samples and, in particular, did not assess polymorphisms associated with DP resistance in southeast Asia, but not to date in Africa.

In conclusion, we found that *P. falciparum* mutations that mediate resistance to SP are common in Uganda, with modest additional selection by IPT with SP, and that use of the promising SP replacement, DP, was associated with selection of mutations that mediate resistance to aminoquinolines. Thus, the benefits of chemoprevention must be balanced with the potential drawback of the selection of drug-resistant malaria parasites.

MATERIALS AND METHODS

Source of samples for study. We utilized *P. falciparum* samples from a randomized, double-blinded trial comparing IPTp with monthly SP versus monthly DP in 782 pregnant women in Busia District, Uganda (17). Briefly, women were randomized to receive either monthly DP or monthly SP beginning at 16 to 20 weeks gestation. Blood samples were obtained before the initiation of IPTp and then during monthly scheduled routine visits and whenever subjects presented with symptomatic malaria.

Identification of *P. falciparum* infections. All blood samples collected from study subjects were assessed for *P. falciparum* by quantitative PCR (qPCR). qPCR targeting the multicopy conserved varATS gene acidic terminal sequence of *P. falciparum* was performed as previously described (32).

Selection of samples for genotyping. Blood samples positive for *P. falciparum* by qPCR were considered for genotyping. For samples collected prior to initiation of IPTp and samples from the SP arm of the study, both of which yielded more positive samples than needed to reach our calculated sample size, genotyping was performed on a randomly chosen subset of samples that were positive by qPCR at parasitemia of at least 10 parasites/μl, as higher parasitemias provide improved genotyping yields. For the DP arm in which a smaller number of samples was *P. falciparum* positive, due to the improved protective efficacy of this regimen, all samples positive by qPCR at parasitemia of at least 1 parasite/μl were genotyped. Considering a sample size formula for two proportions and power (alpha) set at 0.05, the sample size for a 3:1 ratio between control and intervention arms was calculated at 375 samples

before initiation of study drugs and 125 after initiation of SP. Due to a limited number of positive samples, we evaluated all 80 samples from the DP treatment arm with at least 1 parasite/μl.

Characterization of *P. falciparum* genetic polymorphisms. Genomic DNA was extracted from red blood cell pellets using the Invitrogen PureLink genomic DNA minikit following the manufacturer's instructions. *P. falciparum* genetic polymorphisms of interest were characterized by ligase detection reaction-fluorescent microsphere assays, as previously described (33), with minor modification to incorporate nested PCR (34).

Statistical analysis. In our analysis, we assumed that the selective pressure of SP and DP would be the same through the course of IPTp, regardless of the number of months of therapy before sample collection. Data analysis was performed using Stata version 16. The prevalence of genetic polymorphisms of interest before and after exposure to IPTp drugs was compared using a 2-sided Fisher's exact test. Prevalence ratios with 95% confidence intervals were reported. A P value of <0.05 was considered statistically significant.

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