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Selection of Known *Plasmodium falciparum* Resistance-Mediating Polymorphisms by Artemether-Lumefantrine and Amodiaquine–Sulfadoxine-Pyrimethamine but Not Dihydroartemisinin-Piperaquine in Burkina Faso▽

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Artemether-lumefantrine (AL), dihydroartemisinin-piperaquine (DP), and amodiaquine–sulfadoxine-pyrimethamine (AQ-SP) offer excellent antimalarial efficacy but may select for parasite polymorphisms that decrease drug sensitivity. We evaluated the selection of known polymorphisms in genes encoding putative transporters (*pfcrt* and *pfmdr1*) and SP targets (*pfdhfr* and *pfdhps*) in parasites that caused new infections within 42 days of therapy for uncomplicated falciparum malaria in Burkina Faso. In 559 children in 2006, 42-day genotype-uncorrected failures were seen in 31.2% with AL, 11.8% with AQ-SP, and 7.6% with DP. After prior AL therapy, selection of wild-type sequences was seen for K76T in *pfcrt* (72.7% mixed or mutant results pretreatment versus 52.1% in new infections; $P = 0.008$) and N86Y (36.0% versus 18.7%; $P = 0.025$) and Y184F (66.7% versus 45.8%; $P = 0.009$) in *pfmdr1*. After prior AQ-SP therapy, selection of mutant sequences was seen for N51I (30.8% versus 61.5%; $P = 0.05$), C59R (28.2% versus 76.9%; $P = 0.002$), and S108N (30.8% versus 76.9%; $P = 0.005$) in *pfdhfr*. After prior DP therapy, selection was not seen for K76T (72.7% versus 77.8%; $P = 0.96$) in *pfcrt* or N86Y (36.0% versus 33.3%; $P = 0.84$), Y184F (66.7% versus 77.8%; $P = 0.39$), or D1246Y (9.3% versus 0%; $P = 0.42$) in *pfmdr1*. In 378 additional treatments with DP in 2007, 42-day uncorrected failure was seen in 10.9%. After prior DP, selection was again not seen for K76T (66.7% mixed or mutant results versus 59.5%; $P = 0.43$) in *pfcrt* or N86Y (38.7% versus 40.5%; $P = 0.85$), Y184F (67.6% versus 73.0%; $P = 0.54$), or D1246Y (3.6% versus 8.1%; $P = 0.50$) in *pfmdr1*. Despite its chemical similarity, piperaquine did not select for the same polymorphisms as chloroquine or AQ, suggesting different mechanisms of resistance.

Malaria, especially that caused by *Plasmodium falciparum*, is one of the most important infectious diseases in the world, and its control is hampered by increasing resistance of malaria parasites to available drugs (18). Recently, standard therapy for uncomplicated falciparum malaria has changed to artemisinin-based combination therapy (ACT) in nearly all countries in which this disease is endemic (47). ACTs all include a rapid-acting and potent artemisinin derivative combined with a longer-acting partner drug. Optimal ACTs offer excellent efficacy via destruction of most infecting parasites by the fast-acting artemisinin and elimination of any remaining parasites by the long-acting partner drug. However, the extended half-lives of the partner drugs may allow selection for drug-resistant parasites after treatment if new infections occur while these drugs are still circulating. In Africa, one of two ACTs, artemether-lumefantrine (AL) or artesunate-amodiaquine (AS-AQ), is now first-line therapy for uncomplicated falciparum malaria in nearly every country where malaria is endemic. Another ACT, dihydroartemisinin-piperaquine (DP), is a first-line therapy for falciparum malaria in Vietnam and has shown excellent efficacy in Asia (21, 39, 42) and Africa (26–27, 49–50). Piperaquine (PQ) has the longest terminal elimination half-life (~3 to 4 weeks) of the available artemisinin partner drugs (25, 33, 45). This feature leads to lower rates of new infection after treatment with DP than after other ACTs (21, 26, 49–50). However, the long half-life of PQ also suggests that DP may be at high risk for selection of PQ-resistant parasites, as the drug will circulate at low levels for many days after therapy, offering selection for relatively resistant parasites inoculated during subsequent mosquito bites. Indeed, PQ resistance became a major problem after PQ monotherapy was heavily used in China in the 1970s and 1980s, with multiple reports of *in vitro* and clinical drug resistance (3). Considering *in vitro* studies from Africa, parasites were generally quite sensitive to PQ; however, some parasites with 50% inhibitory concentrations (IC_{50}) of >100 nM were identified (1, 4, 32), and concern remains that heavy use of DP may select for PQ-resistant parasites that fail therapy with DP.

A number of mechanisms of resistance of malaria parasites to available drugs are now well characterized (19, 48). Resistance to chloroquine (CQ) is mediated principally by the 76T mutation in the putative drug transporter *pfcrt* (5, 14), and a number of mutations in a second putative transporter, *pfmdr1*, may contribute to the resistant phenotype. The same polymorphisms appear to lead to resistance to AQ, a related aminoquinoline; however, AQ often retains efficacy against CQ-resistant parasites, and it appears that *pfmdr1* mutations play a

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TABLE 1. Outcomes from clinical trials comparing combination therapies in Burkina Faso[a]

Therapy (yr of study)	No. of patients:		No. (%) of patients exhibiting:			
	Enrolled	With outcomes	Early treatment failure	Late clinical failure	Late parasitological failure	Adequate clinical and parasitological response
AQ/SP (2006)	184	169	5 (3.0)	6 (3.6)	9 (5.3)	149 (88.2)
AL (2006)	188	176	2 (1.1)	34 (19.3)	19 (10.8)	121 (68.8)
DP (2006)	187	172	2 (1.2)	8 (4.7)	3 (1.7)	159 (92.4)
DP (2007)	378	367	0	10 (2.7)	30 (8.2)	327 (89.1)

[a] Results are shown for a three-arm comparative trial conducted in 2006 (50) and a single-arm trial of DP conducted in 2007. Results represent genotype-uncorrected clinical outcomes.

more important role in resistance than with CQ (7, 20, 36). PQ is a bisquinoline related to CQ and AQ. As noted above, PQ resistance was noted in China some decades ago; however, molecular mediators of resistance were not reported, and mechanisms of resistance to PQ are unknown. For the antifolate sulfadoxine-pyrimethamine (SP), resistance-mediating polymorphisms are well described, with parasites in Africa commonly containing four to five mutations in the target enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) (19).

A sensitive means of identifying early selection for parasites with decreased sensitivity to antimalarial drugs is to search for the selection of resistance-mediating polymorphisms in parasites that emerge soon after therapy, when ACT partner drugs are still circulating. Such studies in Africa showed that (i) CQ (5, 44), AQ (7, 20, 22–24), and AQ-containing combinations (6, 23, 34) selected for *pfcrt* and *pfmdr1* mutations associated with resistance to CQ and AQ; (ii) AL selected for wild-type sequences at these same loci (8, 24, 29, 41, 51); and (iii) SP or SP-containing combinations selected for known resistance-mediating polymorphisms in *pfdhfr* and *pfdhps* (6, 7, 51). As DP is one of the most promising new antimalarials for Africa, but also might provide the greatest selective pressure for resistance, we studied selection by DP of polymorphisms known to mediate responses to other drugs in *P. falciparum* samples from two clinical trials in Burkina Faso. We found that, although PQ is closely related to CQ and AQ, it did not select for polymorphisms known to mediate diminished sensitivity to the other aminoquinolines.

MATERIALS AND METHODS

Clinical trials. Samples were from two clinical trials, both conducted at three government health dispensaries in Bobo-Dioulasso, Burkina Faso. Results from the first clinical trial have been published, and the prior report includes a detailed description of the study methodology (50). In brief, individuals over 6 months of age with documented uncomplicated falciparum malaria were randomized to receive AL, AQ-SP, or DP and followed for 42 days, and outcomes were classified according to WHO guidelines. The second trial had an identical design, with treatment of patients aged over 6 months for uncomplicated malaria and rigorous 42-day follow-up, except that the trial included only a single study drug, DP, as it was designed to evaluate the pharmacokinetics of this drug. Both trials were registered (ISRCTN94367569 and ISRCTN59761234) and approved by institutional review boards of the Institut de Recherche en Sciences de la Santé/Centre Muraz, Burkina Faso, and the University of California, San Francisco.

Laboratory studies. Blood samples were collected on filter paper on the day of initial diagnosis and for episodes of recurrent parasitemia more than 6 days after the initiation of therapy. DNA was isolated with Chelex resin. For recurrences within 42 days, paired initial and recurrence samples were genotyped in a stepwise fashion by the use of *msp-2*, *msp-1*, and four microsatellites, as previously described (17). If, at any locus, there were no alleles in common between day-0 and the day-of-recurrence samples, the infection was classified as new. If at least one allele was in common at each of the six loci, the infection was classified as a recrudescence.

Relevant resistance-associated single-nucleotide polymorphisms were assessed. Polymorphisms studied were as follows: K76T in *pfcrt*; N86Y, Y184F, and D1246Y in *pfmdr1*; and, for AQ-SP-treated patients, N51I, C59R, and S108N in *pfdhfr* and A437G and K540E in *pfdhps*. All mutations were identified using nested PCR, followed by restriction enzyme digestion, as previously described (10, 12, 13). Digestion products were resolved by gel electrophoresis, and results were classified as wild type or mutant, based on migration patterns. Mixed samples, containing both wild-type and mutant alleles, were classified as mutant. Investigators were blinded to treatment group and outcomes during the molecular analysis.

Statistical analysis. For our first trial, to evaluate differences in prevalences of polymorphisms prior to therapy and in new infections after therapy, we compared prevalences of each polymorphism of interest in 150 (for *pfcrt* and *pfmdr1*) or 39 (for *pfdhfr* and *pfdhps*) randomly selected pretreatment samples with those in parasites that caused new infection within 42 days after a prior therapy. For our second trial, comparisons were made with 111 randomly selected pretreatment samples. All data were entered and verified using SPSS or EpiInfo 6.04 and analyzed using STATA version 8.0. Categorical variables were compared using the chi-squared or Fisher's exact test as appropriate. A P value of <0.05 was considered statistically significant.

RESULTS

Clinical trials providing parasites for study. Two trials from Bobo-Dioulasso, Burkina Faso, provided the parasites evaluated in this study. A trial of 580 patients aged ≥6 months performed in 2006 compared the efficacies of AL, AQ-SP, and DP against uncomplicated falciparum malaria; results of this trial have already been published (50). The trial demonstrated 42-day genotype-uncorrected failures in 55/176 patients (31.2%) with AL, 20/169 (11.8%) with AQ-SP, and 13/172 (7.6%) with DP (Table 1). Clinical failures were primarily due to new infections after therapy. Recrudescences over 42 days after therapy were uncommon (<5% for all groups).

A second trial included 378 treatments for uncomplicated falciparum malaria in patients aged ≥6 months. In this trial, all subjects received DP to allow detailed assessment of drug pharmacokinetics. Patient outcomes were followed over 42 days in the same manner as in our first trial. Pharmacokinetic data will be published separately. This trial demonstrated 42-day genotype-uncorrected failures in 41/378 patients (10.9%) (Table 1). As in the prior trial, recrudescences over 42 days after therapy were uncommon (<1%).

Selection of polymorphisms in parasites causing new infections in a randomized trial of AL, AQ-SP, or DP. We evaluated polymorphisms in *pfcrt* (K76T) and *pfmdr1* (N86Y, Y184F, D1246Y) associated with resistance to CQ and AQ and those in *pfdhfr* (N51I, C59R, S108N) and *pfdhps* (A437G, K540E)

TABLE 2. Selection of polymorphisms in new infections after therapy with AL, AQ/SP, or DP

Gene	SNP[c]	Prevalence (%) in baseline samples[a]	Selection (%) in new infections after prior therapy with[b]:			Selection by[d]:		
			AL (n = 48)	AQ-SP (n = 13)	DP (n = 9)	AL	AQ-SP	DP
pfcrt	K76T	72.7	52.1	76.9	77.8	WT (0.008)	No (0.99)	No (0.96)
pfmdr1	N86Y	36.0	18.7	61.5	33.3	WT (0.025)	No (0.13)	No (0.84)
	Y184F	66.7	45.8	69.2	77.8	WT (0.009)	No (0.91)	No (0.39)
	D1246Y	9.3	6.2	7.7	0	No (0.71)	No (0.76)	No (0.42)
pfdhfr	S108N	30.8		76.9			M (0.005)	
	N51I	30.8		61.5			M (0.05)	
	C59R	28.2		76.9			M (0.002)	
pfdhps	A437G	76.9		69.2			No (0.41)	
	K540E	0		0			No	

[a] n = 150 for *pfcrt* and *pfmdr1*; n = 39 for *pfdhfr* and *pfdhps*.
[b] Proportions of infections classified as mixed or mutant at each allele are shown.
[c] SNP, single-nucleotide polymorphism.
[d] Values indicate whether statistically significant selection was toward the wild-type (WT) sequence or the mutant (M) sequence or whether there was no selection observed (No); for these assessments, *P* values are shown in parentheses.

associated with diminished responses to SP. We did not evaluate three additional mutations (S1034C and N1042D in *pfmdr1*; I164L in *pfdhfr*) or alterations in *pfmdr1* copy number that are important elsewhere but have never, to our knowledge, been seen in Burkina Faso. After prior AL therapy, new infections over 42 days were quite common, likely due to the relatively short half-life of lumefantrine compared to those of other artemisinin partner drugs. As has been reported previously, AL selected for wild-type sequences in *pfcrt* and *pfmdr1* (Table 2). Polymorphisms in which there was statistically significant selection in new infections after prior therapy with AL, all with selection for the wild type, were K76 in *pfcrt* and N86 and Y184 in *pfmdr1*. New infections were uncommon after treatment with AQ-SP or DP, limiting statistical power for identification of selection of polymorphisms in new infections. Polymorphisms in which there was selection for mutant sequences after treatment with AQ-SP were 51I, 59R, and 108N in *pfdhfr*, and there was also a nonsignificant trend toward selection of 86Y in *pfmdr1*. After prior DP, selection was not seen for the tested polymorphisms in *pfcrt* and *pfmdr1*. However, since only nine new infections occurred within 42 days after treatment with DP, statistical power was limited for these analyses.

Selection of polymorphisms in parasites causing new infections after therapy with DP. Since few new infections were available for analysis in our first trial with DP, we analyzed a larger set of 378 treatments from a one-arm efficacy and pharmacokinetic study in Bobo-Dioulasso in 2007. This study confirmed the lack of selection for *pfcrt* and *pfmdr1* polymorphisms seen in the prior trial. The prevalence of 76T in *pfcrt* and 86Y, 184F, and 1246Y in *pfmdr1* was the same in baseline samples and in isolates causing new infections within 42 days after prior therapy with DP (Table 3).

DISCUSSION

Our randomized trial allowed us to evaluate the selective pressures for known resistance-mediating polymorphisms of aminoquinoline and antifolate antimalarial therapies. We found that, as expected based on prior studies, AL selected for wild-type sequences in *pfcrt* and *pfmdr1* and AQ-SP selected for mutant sequences in *pfdhfr*. To our knowledge, the selective pressure of DP has not been studied previously. Surprisingly, PQ, which is closely related to CQ and AQ, did not select for the polymorphisms in *pfcrt* or *pfmdr1* that mediate resistance to and are selected by CQ and AQ. Analysis of additional samples from a second trial of DP yielded similar results. Our results suggest that PQ does not have the same selective pressure, and possibly not the same mechanisms of resistance, as CQ and AQ.

In our two trials, recrudescences after treatment with DP were seen in only 5 of 550 evaluable episodes, and these few recrudescences may have been due to genotyping misclassification and host factors in addition to potential parasite resistance. Thus, there was limited opportunity to identify new mediators of DP resistance by analysis of recrudescent parasites. A more sensitive approach to identify selective pressure is to search for the impacts of treatments on infections that occur soon after prior therapies. In the case of DP, dihydroartemisinin has a half-life of about an hour (43), and so its impact on later infections is expected to be negligible. In con-

TABLE 3. Selection of polymorphisms in new infections after therapy with DP

Gene	SNP	Prevalence (%) in baseline samples (n = 111)	Selection (%) in new infections after prior DP therapy (%)[a]	Selection by DP[b]
pfcrt	K76T	66.7	59.5	No (0.43)
pfmdr1	N86Y	38.7	40.5	No (0.85)
	Y184F	67.6	73.0	No (0.54)
	D1246Y	3.6	8.1	No (0.50)

[a] Proportions of infections classified as mixed or mutant at each allele are shown.
[b] "No" indicates that there was no selection observed; *P* values are shown in parentheses.

trast, the half-life of PQ is ~3 to 4 weeks (25, 33, 45). Thus, infections that emerge over 42 days after therapy necessarily are from parasites that became established despite persisting PQ drug levels, and these parasites can offer clues toward mediators of resistance.

Prior therapy with AQ-SP selected for new infections with the 86Y mutation in *pfmdr1*, but not for other known *pfcrt* and *pfmdr1* mutations. This limited selection might be explained by small sample size, as few new infections were seen after therapy with AQ-SP. AQ-SP also selected for three common mutations in *pfdhfr*, but not another common mutation, 437G in *pfdhps*. These results are consistent with prior data suggesting that the key determinants of resistance to SP are, for *pfdhfr*, 59R, which was selected by AQ-SP, and, for *pfdhps*, 540E, which was not selected, presumably because it is rare in West Africa (9, 38). The absence of parasites with 540E in *pfdhps* in our study likely explains the excellent efficacy of AQ-SP in Burkina Faso (50). AL provided selective pressure in the opposite direction from that of AQ, with selection for wild-type sequences at K76 in *pfcrt* and N86 and Y184 in *pfmdr1*, as has been reported previously (8, 24, 29, 41, 51). These wild-type alleles are associated with diminished sensitivity to halofantrine and mefloquine, which are related to lumefantrine (40, 46). Failures with AL have been uncommon, but it is likely that *pfcrt* and *pfmdr1* wild-type parasites will be more likely than the mutant parasites commonly circulating in Africa to evade the effects of AL.

PQ monotherapy and chemoprophylaxis were heavily used in China beginning in the 1970s, and many reports subsequently noted unacceptable levels of *in vitro* and clinical resistance to PQ (3). Mechanisms of resistance to PQ are unknown. Subsequently, dihydroartemisinin and PQ were combined with other drugs and then developed as a two-drug ACT in the last decade. Recent studies have demonstrated outstanding efficacy against uncomplicated falciparum malaria for DP, with few recrudescences, and a strong posttreatment prophylactic effect, with significantly fewer new infections after therapy than with AS/AQ or AL (21, 26, 27, 49, 50). Thus, DP is one of our most promising ACTs for the control of falciparum malaria. However, the history of widespread resistance to PQ and the strong pressure for the selection of resistant parasites by this long-lasting drug remain a concern. Thus, it was important to explore selection by DP of parasites with potential mediators of resistance.

Our evaluation of over 500 samples from patients treated for falciparum malaria with DP in two trials in Burkina Faso showed that, in contrast to results with CQ and AQ, prior treatment with DP did not select for parasites with *pfcrt* and *pfmdr1* polymorphisms associated with resistance to CQ and AQ. PQ was equally active against parasites sensitive and resistant to CQ in studies from Madagascar (4) and Cameroon (1), although some older studies from China reported cross-resistance between these drugs (3). A recent study of laboratory isolates showed that diminished responsiveness of parasites to PQ was associated with 76T in *pfcrt*, but not with common *pfmdr1* polymorphisms, although the 11 isolates studied had a rather narrow range of sensitivity to PQ (30). A recent study of clinical isolates from Uganda showed correlation between sensitivities to CQ, monodesethylamodiaquine, and quinine, but not PQ (35). Taken together, available results and our new findings suggest that DP does not select as readily as does AQ for known resistance-mediating polymorphisms. PQ may not readily select for these polymorphisms because resistance to PQ is not mediated by them. PQ (molecular weight [MW], 536) is larger than AQ (MW, 465) and CQ (MW, 320), and so it may be unable to utilize the same transporters as the other aminoquinolines to reach its site of action. Alternatively, as the mechanism of action of PQ is unknown, it is possible that the drug acts in a different location than do CQ and AQ and so is unaffected by alterations in *pfcrt* and *pfmdr1*, both of which appear to encode transporters in the food vacuole membrane.

Our results have important implications for the use of new drug regimens to control malaria. At present, AL and AS-AQ are the recommended first-line therapies for uncomplicated falciparum malaria in nearly all countries in Africa. AL offers excellent efficacy, but in high-transmission areas, the relatively short half-life of lumefantrine leads to a high incidence of new infections soon after therapy (26, 49). AS-AQ has shown a significantly lower efficacy than has AL in East Africa (11, 31), presumably due to high prevalence of AQ-resistant parasites in this region. In addition, AQ toxicity has been a concern for many years, and recent reports of frequent neutropenia in HIV-infected (15) or uninfected (2) patients, recurrent neutropenia in children treated repeatedly with AQ-SP (28), and neutropenia and transaminitis in AQ-treated normal volunteers (16, 37) are of concern. DP offers a well-tolerated ACT with excellent efficacy and a long posttreatment prophylactic effect. However, the long terminal elimination half-life of PQ may allow for frequent selection of resistant parasites when new infections occur after dihydroartemisinin has been eliminated but low levels of PQ persist in the bloodstream. The relationship between pharmacokinetics, other factors, and resistance selection is complex (43), and it is unclear if or when PQ resistance may impact upon its antimalarial efficacy in Africa. For the long term, consideration may be given to adding an additional long-acting drug to DP to protect against selection of resistance to PQ. At present, our new results indicate that DP does not readily select for the polymorphisms that play a major role in resistance to CQ and AQ. Thus, DP may continue to show excellent efficacy in the face of increasing resistance to other aminoquinolines, but the mechanism of PQ resistance remains unknown, and we currently are without molecular markers to identify parasites with diminished sensitivity. Thus, surveillance for resistance by *in vitro* assessments of parasite sensitivity to PQ and research to identify mechanisms of PQ resistance are of high priority.

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