Resistance-Mediating *Plasmodium falciparum* pfcrt and pfmdr1 Alleles after Treatment with Artesunate-Amodiaquine in Uganda

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Key parasite polymorphisms were assessed in subjects treated for malaria with artesunate-amodiaquine in Tororo, Uganda. For pfcrt, all of the isolates tested had the CVIET haplotype. For pfmdr1, 86Y and 1246Y were common at baseline and their prevalences were significantly higher in new isolates after therapy, indicating that treatment selected for mutations associated with a decreased response to amodiaquine.

Increasing drug resistance has necessitated changes in antimalarial therapy in Africa (10). Multiple highly effective artemisinin-based combination therapy (ACT) regimens are now available, but the optimal choice for malaria in most areas remains uncertain. All ACTs combine a short-acting artemisinin with a long-acting partner drug, and continued success of these regimens depends on the activity of both component drugs. Prolonged circulation of the partner drugs suggests that selection of resistance to these agents may occur readily. An early warning sign of resistance development may be the selection by therapy of polymorphisms known to be associated with resistance.

One important drug is amodiaquine (AQ), which continues to be used as monotherapy and is a component of two combination regimens now recommended by the WHO, AQ-sulfadoxine-pyrimethemine (SP) and artesunate (AS)-AQ (21). AS-AQ was highly efficacious for the treatment of uncomplicated malaria in Uganda (4, 20, 22) and other African countries (1, 12). The Uganda policy was changed in 2005 to incorporate artesunate-lumefantrine (AL) as the first-line antimalarial therapy available, but the optimal choice for malaria in most areas remains uncertain. All ACTs combine a short-acting artemisinin with a long-acting partner drug, and continued success of these regimens depends on the activity of both component drugs. Prolonged circulation of the partner drugs suggests that selection of resistance to these agents may occur readily. An early warning sign of resistance development may be the selection by therapy of polymorphisms known to be associated with resistance.

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Mechanisms of resistance to AQ are incompletely understood. The mechanism is likely similar to that of chloroquine (CQ), as cross-resistance is well described (14) and CQ-resistant strains displayed reduced accumulation of AQ (3). However, AQ and its metabolites are active against many CQ-resistant parasites (14) and its superiority is demonstrated by the markedly superior antimalarial efficacy of AQ-SP over that of CQ-SP (20). Resistance to CQ is principally mediated by a single mutation, K76T, in the *Plasmodium falciparum* CQ resistance transporter gene (*pfcr*). Additional *pfcr* polymorphisms probably improve the fitness of resistant parasites. Among these, a set adjacent to residue 76 has been used to assign a haplotype to characterize parasites from different locales (9). Polymorphisms in a second putative transporter, *P. falciparum* multidrug resistance 1 (*pfmdr1*), may affect responses to a number of drugs. The 86Y and 1246Y mutations have been associated with diminished responses to CQ and AQ (15, 17), and these mutations were also selected for by AQ therapy in Burkina Faso (6). In contrast, N86, D1246, and other wild-type alleles are associated with diminished in vitro responses to a number of drugs, including mefloquine, halofantrine, quinine, and artemisinin (15, 17). In Tanzania (19), Uganda (7), and Burkina Faso (23), use of AL selected for reversion to wild-type alleles, probably because of the selective pressure of lumefantrine. Thus, drugs may select for parasites more or less resistant to other agents. In this setting, we were interested in the impact of AS-AQ on mutations associated with altered responses to commonly used drugs and so studied its selection of key *pfcrt* and *pfmdr1* polymorphisms.

This study utilized samples from a clinical trial comparing the efficacies of AL and AS-AQ in Tororo, Uganda, a region of very high malaria transmission (4). Briefly, children 1 to 10 years old with uncomplicated malaria were randomly assigned to receive directly observed therapy with one of the ACTs and were monitored for 28 days. Treatment responses were monitored on the basis of WHO criteria. For episodes of recurrent parasitemia that occurred more than 3 days after therapy, polymorphisms in merozoite surface protein 1 (MSP-1) and MSP-2 were compared to distinguish recrudescence from new infections, as previously described (5). Outcomes were classified as recrudescence if all of the MSP alleles present on the day of failure were present on the day of enrollment and new infections if new alleles had arisen. The clinical trial was approved by the Uganda National Council of Science and Technology and the Institutional Review Boards of Makerere University; the University of California, San Francisco; and the University of California, Berkeley.

Our clinical trial identified no recrudescences but frequent new infections (66% of subjects) within a month after treatment of uncomplicated malaria with AS-AQ. We evaluated polymorphisms at *pfmdr1* alleles N86Y, Y184F, S1034C, N1042D, and D1246Y in 201 isolates collected before the initiation of treatment (representing all of the patients randomly assigned to therapy with AS-AQ) and 132 isolates from all patients with new infections that presented over 28 days of follow-up after therapy. Blood was collected on filter paper on the day of initial diagnosis and at the time of new infection. DNA was isolated by Chelex extraction (16). Alleles were identified by the nested-PCR and restriction fragment length

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polymorphism methods as previously described (8). Digestion products were resolved by gel electrophoresis, and results were classified as wild type, mutant, or mixed on the basis of the migration patterns of ethidium bromide-stained fragments. We also evaluated \textit{pfcr} haplotypes at positions 72 to 76 in 90 randomly selected pretreatment and 90 randomly selected new-infection isolates by using methods described at http://medschool.umaryland.edu/cvd/2002_pcr_asra.asp. Data were entered and verified by using SPSS and analyzed by using STATA 8.0. The prevalences of mutations in pretreatment and new-infection isolates were compared by using the chi-square or Fisher exact test, as appropriate. A \( P \) value of <0.05 was considered statistically significant.

Genotyping was successful for all isolates. For \textit{pfmdr1}, the prevalence of mutant alleles (including mixed and pure mutant isolates) increased significantly from pretreatment to new-infection isolates for 86Y (182/201, 90.5%, to 128/132, 97.0%; \( P = 0.03 \), 1246Y (167/201, 83.1%, to 120/132, 90.9%; \( P = 0.04 \)), and these two mutations together (164/201, 81.6%, to 119/132, 90.2%; \( P = 0.03 \); Fig. 1). In contrast, the prevalence of wild-type allele Y184 increased from pretreatment to new-infection isolates (171/201, 85.1%, to 122/132, 92.4%; \( P = 0.04 \)). Only wild-type alleles were seen at positions 1034 and 1042. For \textit{pfcr}, the CVIET haplotype at positions 72 to 76 was seen in all 180 samples analyzed.

Isolates from our study had a high prevalence of \textit{pfmdr1} 86Y and 1246Y mutations, and the prevalence was greater in new isolates after therapy with AS-AQ than in isolates collected before therapy. In particular, consistent with studies of AQ monotherapy (6, 11) from other regions, the AQ-containing regimen selected for the 86Y mutation. In Burkina Faso, the presence of the 86Y mutation prior to treatment predicted failure of AQ monotherapy, suggesting that selection of mutant parasites will lead to an increased likelihood of drug resistance. Another study, in Sudan, did not identify associations between the 86Y allele and AQ treatment outcomes, but that study assessed only 14-day treatment outcomes and did not evaluate selection of mutant parasites after therapy (13).

As the \textit{pfcr} 76T mutation that mediates CQ resistance was universal in our isolates, we could not identify associations between \textit{pfcr} alleles and AQ treatment outcomes, as seen at sites with a lower prevalence of the mutant parasites, where \textit{pfcr} 76T predicted AQ treatment failure (6, 13). It was also of interest to characterize \textit{pfcr} haplotypes. Molecular studies have shown that parasites transfected with \textit{pfcr} with the SVMNT haplotype have decreased sensitivity to AQ and its active metabolite (18), so if parasites of this haplotype are circulating, as was recently demonstrated in Tanzania (2), they might be selected by AS-AQ. However, all of our isolates contained the CVIET haplotype, suggesting that selection of other \textit{pfcr} haplotypes is not yet ongoing in Tororo.

Our study adds to our understanding of the impacts of current antimalarial drugs on genotypes that affect treatment outcomes. Recent studies in Tanzania (19), Uganda (7), and Burkina Faso (23) showed selection of the \textit{pfmdr1} wild-type N86 and/or D1246 alleles after treatment with AL, presumably because of the selective pressure of lumefantrine. Our study and another recent report (6) show the opposite selection with AS-AQ. Thus, different ACTs exert opposite selective pressures, in manners that may increase or decrease the sensitivity of parasites to widely used drugs. In this study, all changes in allele prevalence were modest, probably because of sample size limitations, a baseline high prevalence of key mutations, and potential influences of other, as-yet-unidentified, genetic markers. Better characterization of the selective pressures of different antimalarials and their impacts on outcomes is needed.

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