MAJOR ARTICLE

Selection of Parasites with Diminished Drug Susceptibility by Amodiaquine-Containing Antimalarial Regimens in Uganda

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Background. Amodiaquine (AQ) is paired with artesunate (AS) or sulfadoxine-pyrimethamine (SP) in recommended antimalarial regimens. It is unclear how readily AQ resistance will be selected with combination chemotherapy.

Methods. We collected 61 *Plasmodium falciparum* samples from a cohort of Ugandan children randomized for treatment with AQ-SP, AS-AQ, or artemether-lumefantrine (AL) for uncomplicated malaria. In vitro susceptibility to monodesethylamodiaquine (MDAQ) was measured with a histidine-rich protein 2–based enzyme-linked immunosorbent assay, and potential resistance-mediating polymorphisms in *pfmdr1* were evaluated.

Results. Parasites collected from patients treated with AQ-SP or AS-AQ within the prior 12 weeks were less susceptible to MDAQ ($n = 18$; mean of the median inhibitory concentration [IC_{50}], 62.9 nmol/L; range, 12.7–158.3 nmol/L) than were parasites from those not treated within 12 weeks ($n = 43$; mean IC_{50}, 37.5 nmol/L; range, 6.3–184.7 nmol/L; $P = .009$) or only from those patients in the treatment arm that did not receive AQ ($n = 20$; mean IC_{50}, 28.8 nmol/L; range, 6.3–121.8 nmol/L; $P = .004$). The proportion of strains with polymorphisms expected to mediate diminished response to AQ (*pfmdr1* 86Y and 1246Y) increased after AQ therapy, although differences were not statistically significant.

Conclusions. Prior therapy selected for diminished response to MDAQ, which suggests that AQ-containing regimens may rapidly lose efficacy in Africa. The mechanism of diminished MDAQ response is not fully explained by known mutations in *pfmdr1*.

The control of *Plasmodium falciparum* malaria is seriously challenged by resistance to many available drugs [1]. Among those drugs with diminished antimalarial activity are the 4-aminoquinolines, notably chloroquine (CQ) and amodiaquine (AQ). Despite very high levels of resistance to CQ in Africa, AQ often offers effective antimalarial treatment, and it remains an important part of our antimalarial drug portfolio [2]. With increasing resistance, there is now a clear consensus that uncomplicated falciparum malaria should be treated with drug combinations, ideally artemisinin-based combination therapy (ACT) [3]. ACT regimens all rely on an artemisinin component and a longer-acting partner drug [4]. AQ plays a key role in current treatment strategies. When partnered with artesunate (AS), AQ has shown excellent antimalarial efficacy in multiple studies in Africa, and this combination regimen is now available as a coformulated drug [5–9]. In addition, the older combination of AQ plus sulfadoxine-pyrimethamine (SP) has shown good efficacy in many areas [5, 10, 11] and is recommended by the World Health Organization (WHO) to treat uncomplicated malaria when the efficacy of the individual components is acceptable and ACTs are not available [2].

With all ACTs, there is a risk of selection of resistance to artemisinin partner drugs, which circulate long after elimination of the short-acting artemisinin component

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[12]. This is a particular concern for AQ because resistance to this drug is already well established in parts of Africa [8, 13, 14]. AQ appears to act principally after conversion to an active metabolite, monodesethylamodiaquine (MDAQ) [15]; parasites are ~3-fold less susceptible in vitro to MDAQ than to the parent compound [16]. Parasites with a range of in vitro susceptibility to MDAQ have been described in Africa [17–21]. In general, >90% of isolates from different sites in Africa were classified as susceptible to MDAQ (using the median inhibitory concentration [IC_{50}] cutoff of 60 nmol/L MDAQ), although a recent study from Kenya reported that 26% of baseline isolates had an IC_{50} for MDAQ >60 nmol/L [21]. In South America and Asia, resistance to MDAQ or AQ has been more common [16, 22–25]. Relatively little information on *P. falciparum* susceptibility to MDAQ is available from East Africa, the location of our study and an area where resistance to AQ is common.

The mechanism of *P. falciparum* resistance to AQ or MDAQ is incompletely understood; mutations in genes that encode 2 putative drug transporters, *pfcrt* and *pfmdr1*, appear to play important roles. The *pfcrt* 76T mutation is the principal mediator of resistance to CQ [26]. In areas where parasites with wild-type *pfcrt* still circulate, the 76T mutation predicted poor response to therapy with AQ [27–29], and this mutation was selected in parasites that caused recurrent infections after treatment with AQ [11, 28–31]. The *pfcrt* 76T mutation was also associated with decreased in vitro susceptibility to MDAQ in field isolates [32] and in genetically altered laboratory strains [33]. Polymorphisms in a second putative transporter, *pfmdr1*, affect the susceptibility of malaria parasites to a number of drugs [34]. The *pfmdr1* 86Y polymorphism predicted poor AQ treatment outcome in most [28, 29] but not all [27] studies from Africa and was selected by prior therapy with AQ in a number of studies [11, 28–31, 35]. However, prevalence of the *pfmdr1* 86Y mutation was not associated with in vitro drug susceptibility in a study from Africa [32], and the *pfmdr1* 86Y mutation was seen in only ~10% of strains with in vitro resistance to MDAQ in samples from Colombia [22]. Taken together, recent studies suggest that the molecular basis of resistance to AQ is complex, with apparent contributions of polymorphisms in *pfcrt* and *pfmdr1* and likely additional factors that contribute to the resistant phenotype.

As we move to routine treatment of malaria with combination regimens, it is unclear how readily resistance to AQ will be selected by treatment with AQ-containing combinations. To gain insight in this area, we evaluated the in vitro activity of MDAQ against 61 clinical samples from Ugandan children treated with 1 of 3 combination regimens, 2 of which included AQ. We then searched for associations between in vitro drug susceptibility, clinical outcomes, prior drug use, and known molecular mediators of resistance. Most notably, we found that prior use of AQ-containing combinations selected for subsequent infections with diminished responsiveness to MDAQ.

METHODS

Clinical trial. All samples were taken from a cohort of 601 children randomly selected from a community in Kampala, Uganda, aged 1–10 years at enrollment during 2004–2005, as previously described [9, 36]. Upon presentation with the first episode of uncomplicated malaria, participants were randomly assigned to receive AQ-SP, AS-AQ, or artemether-lumefantrine (AL), which they received thereafter for each episode of uncomplicated malaria. With each treatment, efficacy was assessed following WHO guidelines [37], with genotyping to distinguish recrudescence and new infection after therapy, all as described elsewhere [9]. For this study, we analyzed a subset of samples collected from August 2006 through March 2007 for which parasites were successfully grown in short-term culture to allow determination of in vitro drug susceptibility. The clinical trial was approved by the Uganda National Council of Science and Technology and the Institutional Review Boards of Makerere University and the University of California, San Francisco.

In vitro susceptibility to MDAQ. After diagnosis of uncomplicated malaria and before the initiation of therapy, blood was collected into heparinized tubes and delivered within 30 min to our laboratory. Specimens were centrifuged, and supernatant and buffy coat suspension were removed. Erythrocytes were washed twice in RPMI 1640 medium prewarmed to 37°C, and samples were diluted to 0.05% parasitemia and 2% hematocrit. Parasites were assessed for drug susceptibility under sterile conditions in RPMI 1640 supplemented with 0.5% Albumax and 100 μg/mL gentamicin in 96 well-cell culture plates that had been coated with 7 serial dilutions of MDAQ (6.25–400 nmol/L, each in duplicate) and dried overnight. Wells without drugs served as controls. For each sample, 200 μL of culture was added to each well, with duplicate wells for each concentration of MDAQ. Plates were incubated for 72 h at 37°C in a candle jar, and samples were then frozen (−20°C overnight) and thawed before analysis. Parasite growth was assayed by comparing levels of histidine-rich protein 2 (HRP-2) in treated and control cultures [38]. HRP-2 was quantified by using a commercial ELISA test kit (Malaria Antigen CELISA; Cellabs). Samples were diluted (1:4–1:10, the same dilution for each sample in an experiment) in water, and 100 μL of each hemolyzed sample preparation was added to an ELISA plate precoated with anti–HRP-2 and incubated at room temperature for 1 h. Plates were then washed 4 times with the kit washing solution, 100 μL of secondary antibody was added to each well for 1 h at room temperature, plates were again washed 4 times, 100 μL of chromogen substrate was added to each well, plates were incubated for 15 min in the dark, and 50 μL of stopping solution was added. Absorbance (450 nm) was then measured

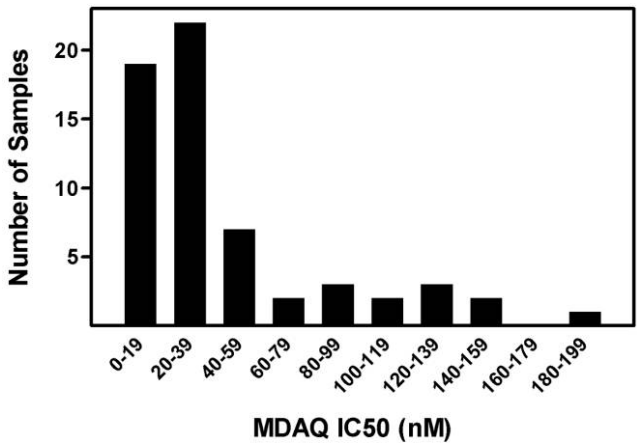

Figure 1. Sensitivity of clinical samples to monodesethylamodiaquine (MDAQ). Parasites were placed into culture, and susceptibilities were assessed by using an enzyme-linked immunosorbent assay to compare levels of histidine-rich protein 2 in treated samples with those in untreated control samples.

for each well with a SpectraMAX 340 spectrophotometer (Molecular Devices). Optical density values were fitted to normal curves that were based on serial dilutions of HRP-2 standards and a 4-parameter curve model (Softmax Pro software version 2.1.1; Molecular Devices), and IC_{50} values were calculated by using a nonlinear regression model, with attention to test validity based on adequate readings above background, as described elsewhere [39].

***Genetic polymorphisms in* pfmdr1.** After diagnosis of uncomplicated malaria, blood spots were also dried on filter paper, which was stored with desiccant in sealed plastic bags at room temperature. DNA was subsequently extracted with chelex, and previously characterized polymorphisms in *pfcrt* and *pfmdr1* were analyzed by nested polymerase chain reaction, followed by mutation-specific restriction endonuclease (*Apo*I, *Afl*III, *Dra*I, *Dde*I, *Ase*I, and *Eco*RV) cleavage, as previously described for the *pfcrt* K76T and *pfmdr1* N86Y, Y184F, S1034C, N1042D, and D1246Y polymorphisms, respectively [40]. DNA fragments were separated by agarose gel electrophoresis, and electrophoretic band patterns were categorized as wild type, mixed, or mutant genotypes by visual inspection of gels and comparison with DNA from control strains 7G8 and FCR3, obtained from the Malaria Research and Reference Reagent Resource Center (MR4).

Statistical analyses. Clinical data were entered and verified with Access software (Microsoft). Statistical analyses were performed with Stata software, version 10 (StataCorp). Clinical outcomes were assessed as described elsewhere [9]. Values for in vitro drug susceptibility of parasite samples were not normally distributed and were assessed with the Kruskal-Wallis rank test. Prevalences of mutations were compared with the Fisher exact test. For all assessments, $P < .05$ was considered statistically significant.

RESULTS

In vitro drug susceptibility of fresh* P. falciparum *samples. Blood was collected from children who presented with uncomplicated falciparum malaria, and the in vitro susceptibility of infecting parasites to MDAQ (the principal active metabolite of AQ) was determined. Of 72 samples cultured during the time frame of the study, 61 had successful in vitro analyses, with a wide range of in vitro drug susceptibility (Figure 1, Table 1). Samples that could not be evaluated included 1 that was contaminated with bacteria, 4 that did not grow in culture, and 6 that failed to provide an adequate dose response curve for analysis. Using a previously assigned cutoff for resistance of $IC_{50} > 60$ nmol/L [17], 13 (21.3%) of the 61 parasite samples were categorized as resistant to MDAQ, and 8 (13.1%) had $IC_{50} > 100$ nmol/L. Control parasite lines (reference numbers from MR4 are provided) yielded IC_{50} results of 30.9 nmol/L for D6 (MRA-285), 29.8 nmol/L for HB3 (MRA-155), 267.7 nmol/L for W2 (MRA-157), and 262.1 nmol/L for K1 (MRA-159); the first 2 strains are considered susceptible to and the last 2 are considered resistant to CQ.

Association of in vitro drug susceptibility and clinical treatment failure. The 3 combination regimens included in our clinical trial were all efficacious, and therefore few recrudescences occurred after therapy [9]. Only 7 (11.5%) of the 61 samples were from episodes of malaria classified as recrudescent. For these samples, and for both the AQ-containing treatment arms and the AL arm, recrudescence was equally likely in those with parasites susceptible and resistant to MDAQ (Table 2). Thus, although sample size was small for this analysis, we saw no clear association between MDAQ susceptibility and treatment failure.

Parasites with diminished susceptibility to MDAQ were selected by prior therapy with AQ. To assess the effect of recent prior therapy on drug susceptibility, we compared the MDAQ susceptibility of parasites from patients who were previously treated with AQ-SP or AS-AQ within 12 weeks with those from patients not treated with these drugs within this interval and also with only those from patients in the AL treatment arm, and thus not treated with AQ during the course of the study. Parasites from patients who were previously treated with AQ-SP or AS-AQ within 12 weeks were less sensitive to AQ ($n = 18$; mean IC_{50}, 62.9 nmol/L; range, 12.7–158.3 nmol/L),

Table 1. Summary Information for Parasite Samples

This table is available in its entirety in the online version of *Journal of Infectious Diseases*.

Table 2. Clinical Outcomes Stratified by In Vitro Monodesethylamodiaquine (MDAQ) Sensitivity

MDAQ susceptibility (IC_{50}, nmol/L)	Isolates with indicated susceptibility, no.	Isolates with recrudescent outcomes, no. (%)
AQ-SP		
<60	10	2 (20)
⩾60	5	1 (20)
AQ-AS		
<60	18	1 (5.6)
⩾60	6	0
AL		
<60	20	2 (10)
⩾60	2	1 (50)

NOTE. AL, artemether-lumefantrine; AQ, amodiaquine; AS, artesunate; IC_{50}, median inhibitory concentration; SP, sulfadoxine-pyrimethamine.

compared with parasites from those not treated with an AQ-containing regimen within 12 weeks ($n = 43$; mean IC_{50}, 37.5 nmol/L; range, 6.3–184.7 nmol/L; $P = .008$) or compared with parasites from those in the treatment arm that did not contain AQ (AL subjects; $n = 20$; mean IC_{50}, 28.8 nmol/L; range, 6.3–121.8 nmol/L; $P = .004$) (Figure 2). Similar associations were seen when drug sensitivities were compared only for parasites causing new infections (not shown) and for infections occurring within 6, 8, or 10 weeks of prior therapy; however, differences in drug sensitivities were not statistically significant for intervals ⩽6 weeks (Table 3).

Association of parasite genetic polymorphisms with prior therapy with AQ. We also characterized genetic polymorphisms that have previously been identified in the *pfcrt* and *pfmdr1* genes. Consistent with other results from Kampala [41], all 61 samples that provided in vitro susceptibility results had only the mutant *pfcrt* allele (76T) that has previously been associated with CQ resistance [26]. As seen previously [11, 28–31, 35], prior treatment with AQ (within 12 weeks) was associated with 2 *pfmdr1* polymorphisms, 86Y and 1246Y, that have been associated with decreased responsiveness to this drug, although baseline prevalences of these polymorphisms were high, and differences were not statistically significant (Figure 3). Another polymorphism, 184F, was not selected by prior therapy with AQ, and 2 other mutations—1034C and 1042D—were not seen in any samples. Of note, the lower prevalence of *pfmdr1* 86Y in subjects from the AL treatment group was probably attributable, in part, to selection of the wild-type sequence by AL, as has been shown previously.

Association of parasite genetic polymorphisms and MDAQ susceptibility. We also attempted to identify associations between *pfmdr1* polymorphisms and in vitro susceptibility to MDAQ. Straightforward correlations were not seen (Figure 4). The prevalence of the mutant allele was slightly higher among parasites categorized as resistant for N86Y (prevalence 86Y 38 [79.2%] of 48 in susceptible isolates versus 11 [84.6%] of 13 in resistant isolates), prevalences were the same for 1246Y (prevalence 1246Y 37 [77.1%] of 48 in sensitive versus 10 [76.9%] 13 in resistant), and the prevalence of the mutant allele was lower among resistant parasites for Y184F (prevalence 184F 9 [18.8%] of 48 in sensitive versus 1 [7.7%] of 13 in resistant). No differences in allele prevalence were statistically significant.

DISCUSSION

ACT is now the international standard for the treatment of *P. falciparum* malaria, because multiple new combination regimens offer excellent antimalarial efficacy [4]. However, there is concern that, because all ACTs include a short-acting artemisinin and a long-acting partner drug, the regimens will select for resistance to partner drugs, especially in areas where reinfection after treatment is common. This concern is arguably greatest for AQ-containing combinations, because resistance to this drug is already fairly common. AQ is rapidly metabolized

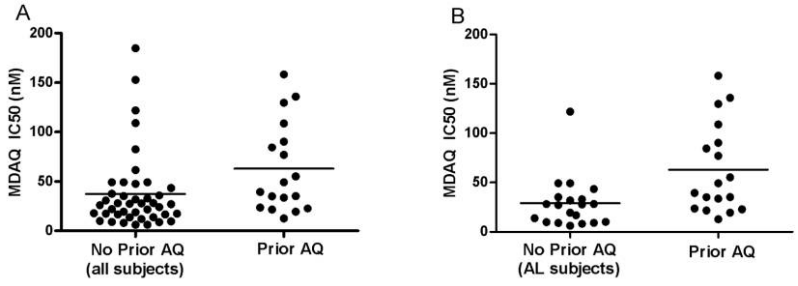

Figure 2. Selection of parasites with diminished response to amodiaquine (AQ). Susceptibilities were determined as described in Methods. Results are plotted for parasites from all patients who did not receive an AQ-containing regimen within 12 weeks (no prior AQ) (*A*) or only those patients in the AL treatment arm who did not receive an AQ-containing regimen for the course of the study (no prior AQ) (*B*). Results for both groups were compared with results for patients who did receive an AQ-containing regimen within 12 weeks prior to the time of this analysis (prior AQ). Mean values are indicated by horizontal lines. Differences for both comparisons were statistically significant, as described in the text. IC_{50}, median inhibitory concentration; MDAQ, monodesethylamodiaquine.

Table 3. Monodesethylamodiaquine (MDAQ) Susceptibility of Parasites Causing Infections in Patients with Recent Infections

Time since prior treatment with AQ, wk	Mean MDAQ IC_{50}, nmol/L	*n*	*P*
6	46.4	11	.076
8	54.5	14	.019
10	57.3	17	.007
12	62.9	18	.004

NOTE. Mean values for the median inhibitory concentration (IC_{50}) were compared with those for samples from patients in the AL treatment arm ($n = 20$; mean IC_{50}, 28.8 nmol/L) by using the Kruskal-Wallis test. AL, artemether-lumefantrine; AQ, amodiaquine.

to MDAQ [15], an active metabolite that has a half-life of ~2 weeks [12]. Thus, after therapy with a regimen containing AQ, MDAQ circulates at decreasing levels for many weeks. We hypothesized that children treated with AQ-containing regimens would be at increased risk of AQ resistance in subsequent infections. To test this hypothesis, we studied susceptibility to MDAQ in *P. falciparum* parasites causing uncomplicated malaria in a cohort of children in Kampala who received AQ-SP, AS-AQ, or AL for each episode of uncomplicated malaria. Kampala is known to have a fairly high prevalence of AQ-resistant malaria [42], and parasites that caused malaria in our cohort demonstrated a wide range of sensitivities to MDAQ. Importantly, parasites that caused infections within 12 weeks of a prior treatment with AQ had decreased susceptibility to MDAQ, compared with those that caused infections in individuals not recently treated with AQ. Thus, as we hypothesized, prior treatment with AQ selected for parasites with diminished drug susceptibility. This result suggests that resistance to AQ could rapidly be selected by treatment of malaria with combinations that include AQ.

We identified a broad range of susceptibilities to MDAQ in parasites causing uncomplicated malaria in our cohort of Ugandan children. Resistant parasites (based on a cutoff of MDAQ $IC_{50} > 60$ nmol/L) were seen in 21% of infections, which was consistent with prior clinical trials in Kampala that showed frequent treatment failures with AQ monotherapy [42]. These results and recent similar findings from Kenya (26% of isolates had MDAQ $IC_{50} > 60$ nmol/L) [21] suggest a more extensive problem with AQ resistance in East Africa than in some other regions of Africa; >90% of parasites were susceptible to MDAQ in vitro in recent studies from Madagascar [43], Ghana [44], Cameroon [17], Congo [20], Senegal [18], and Rwanda [45], as well as in a study involving a collection of isolates from multiple African countries [19]. Older studies that considered susceptibility to AQ (rather than to MDAQ) also generally reported high levels of susceptibility, although 16% of samples were reported to demonstrate in vitro resistance in a study from the Central African Republic [46]. In our study, MDAQ-resistant parasites caused infections in those with and in those without recent prior therapy with AQ; selective pressure from circulating AQ was not required for infection with a resistant strain. However, prior therapy with AQ led to an increased predilection for infection with MDAQ-resistant parasites.

It was also of interest to determine whether the in vitro susceptibility of parasites to MDAQ was associated with clinical outcomes. However, of the 61 parasite samples studied, only 7 represented recrudescent infections as determined with multilocus genotyping, and only 4 of these infections were in subjects in an AQ-containing treatment arm. Therefore, we lacked the power to assess associations between in vitro susceptibility to MDAQ and treatment outcomes. Nonetheless, recrudescences occurred after infection with parasites sensitive and resistant to MDAQ, arguing against any simple association between in vitro drug susceptibility and clinical response. Similarly, in studies from Gabon [47] and Kenya [21], in vitro susceptibility to MDAQ did not correlate with clinical response to therapy with AQ. Indeed, it is likely that clinical responses to AQ-containing combination regimens are complex, with effects of varied pharmacokinetics [15], pharmacogenomics [48], host immunity, and other factors in addition to the drug responsiveness of parasites. Nonetheless, parasite drug susceptibility is clearly an important component of a successful treatment response. A better appreciation of mediators of parasite resistance to AQ will be of value in optimizing the use of available drug regimens.

We also searched for associations between in vitro drug susceptibility and polymorphisms that have previously been linked

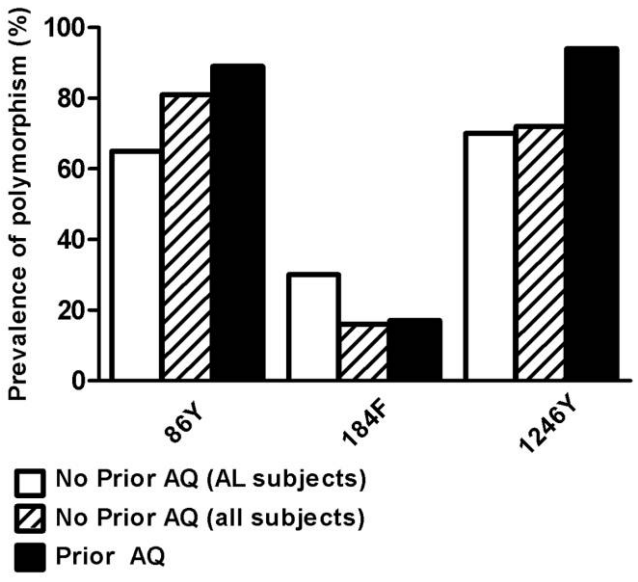

Figure 3. Selection of *pfmdr1* alleles. The proportions of samples with mutations at the alleles indicated are shown for the 2 comparisons described in Figure 2. AL, artemether-lumefantrine; AQ, amodiaquine.

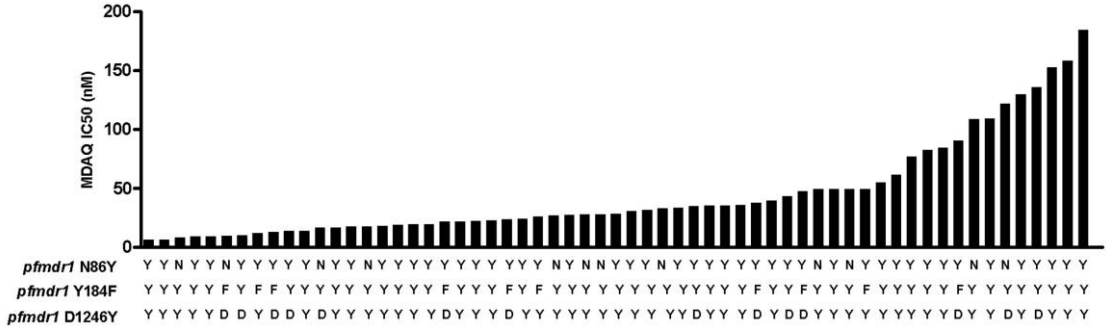

Figure 4. Correlation of in vitro susceptibility to monodesethylamodiaquine (MDAQ) with *pfmdr1* genotypes. For each sample studied, the sequence at the 3 alleles of interest and the in vitro sensitivity to MDAQ are shown.

to altered susceptibility to AQ. The 76T polymorphism in the putative drug transporter *pfcrt*, which is the primary mediator of resistance to chloroquine, also predicts poor response to AQ [27–29] and is selected in new infections after therapy with AQ [11, 28–31]. The *pfcrt* 76T mutation was also associated with decreased in vitro susceptibility to MDAQ in field isolates from the Central African Republic [32] and in genetically altered laboratory strains [33]. Considering our results, it is noteworthy that—even with 100% prevalence of 76T in our set of parasites—79% of samples demonstrated sensitive in vitro responses to MDAQ. Thus, the common *pfcrt* polymorphism, although predictive of decreased response to MDAQ, does not by itself dictate MDAQ resistance. Polymorphisms in a second putative drug transporter, *pfmdr1*, have not been as clearly linked to AQ treatment outcome, but in some studies from Africa, the *pfmdr1* 86Y polymorphism predicted poor AQ treatment outcomes [28, 29]. Also, as seen in this study, *pfmdr1* 86Y was selected by prior therapy with AQ in a number of studies [11, 28–31, 35]. However, prevalence of the *pfmdr1* 86Y mutation was not associated with in vitro drug susceptibility in samples from the Central African Republic [32], from Colombia [22], or in our study from Uganda. Taken together, our results are consistent with those from other areas, which suggest contributions of polymorphisms in both *pfcrt* and *pfmdr1* to AQ resistance, but the likely involvement of additional host (eg, genetics, pharmacokinetics, immunity) and parasite (eg, additional polymorphisms) factors in high level AQ resistance.

Our study had some limitations. Our evaluations of in vitro susceptibility of clinical samples were necessarily limited to parasites capable of growing in short-term culture to allow measurement of in vitro drug susceptibility. Exclusion of parasites that did not grow in culture may have introduced bias. Infections in Kampala are commonly polyclonal, and both complexity of infection [49] and key polymorphisms in *pfmdr1* [50] have been seen to change after in vitro culture. Most of our in vitro susceptibility measurements were thus based on an assessment of a mixed population of parasites in which competition between susceptible and resistant strains might obscure results for parasites capable of mediating resistant outcomes. Comparisons with results from other studies must take into account the fact that a number of different assays have been used to measure in vitro drug susceptibility; comparisons of values between studies may be misleading. In vitro measurements leave potential for error; because they are measured only during the first life cycle after parasite collection, they cannot be repeated. Thus, for any single measurement, there is the possibility that human error or other factors led to misrepresentation of the true results. Despite these limitations, considering our large set of evaluated samples, our results strongly suggest that prior therapy with AQ selects for decreased susceptibility to the drug.

Our results add concern regarding the long-term prospects for AQ-containing combination therapies for the treatment of *P. falciparum* malaria. Selection of resistant parasites was readily apparent after prior therapy with AQ. These results suggest diminishing efficacy of AQ-containing combination regimens as they are increasingly used in Africa. The results further highlight the need for continued scrutiny of the efficacies of current antimalarial regimens, as efficacies will likely diminish over time, and for continued efforts to develop new antimalarial treatments that circumvent drug resistance.

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