In Vivo and In Vitro Efficacy of Amodiaquine against *Plasmodium falciparum* in an Area of Continued Use of 4-Aminoquinolines in East Africa

Philip Sasi,1,2 Abdi Abdulrahman,1,3 Leah Mwai,1 Steven Murithi,1 Judith Straimer,5 Elise Schieck,1,3 Anja Rippert,5 Mahfudh Bashraheil,1 Amina Salim,1 Judith Peshu,1 Ken Awuondo,1 Brett Lowe,1 Munir Pirmohamed,4 Peter Winstanley,4 Steve Ward,4 Alexis Nzila,1 and Steffen Borrmann1,5

1Kenya Medical Research Institute/Wellcome Trust Research Programme, Center for Geographic Medicine Research–Coast, Kilifi, Kenya; 2Department of Clinical Pharmacology, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania; 3Wellcome Centre for Molecular Parasitology, University of Glasgow, Glasgow, and 4University of Liverpool, Liverpool, United Kingdom; 5Institute of Hygiene, University of Heidelberg School of Medicine, Germany

In light of reports of increasing resistance of parasites to amodiaquine in African countries in which *Plasmodium falciparum* is endemic as well as the paucity of recent in vitro sensitivity data, we assessed the in vivo and in vitro sensitivity to amodiaquine of *P. falciparum* isolates from 128 pediatric outpatients (0.5–10 years old) in Pingilikani, Kilifi District, Kenya, who were treated with amodiaquine (10 mg/kg/day for 3 days). The polymerase chain reaction–corrected parasitological cure rate on day 28 (by Kaplan-Meier analysis) was 82% (95% confidence interval [CI], 74%–88%). Twenty-six percent (17/66) of tested pretreatment *P. falciparum* field isolates had 50% in vitro growth inhibition at concentrations of N-desethyl-amodiaquine (DEAQ)—the major biologically active metabolite of amodiaquine—above the proposed resistance threshold of 60 nmol/L, but baseline median DEAQ 50% inhibitory concentration values were not associated with subsequent risk of asexual parasite recrudescence (29 nmol/L [95% CI, 23–170 nmol/L] and 34 nmol/L [95% CI, 30–46 nmol/L] for patients with and those without recrudescences, respectively). The median absolute neutrophil count dropped by 1.3 \( \times \) \( 10^3 \) cells/\( \mu L \) (95% CI, \(- 1.7 \times 10^3 \) to \(- 0.7 \times 10^3 \) cells/\( \mu L \)) between days 0 and 28. The high prevalence of in vitro and in vivo resistance precludes the use of amodiaquine on its own as second-line treatment. These findings also suggest that the value of amodiaquine combinations as first- or second-line treatment in areas with similar patterns of 4-aminoquinoline resistance should be reassessed.

Because of rare but serious hematological and hepatic toxicity when used for malaria prophylaxis [1, 2], amodiaquine is recommended only for treatment of falciparum malaria [3, 4]. There was a renewed interest in amodiaquine monotherapy in the face of high chloroquine resistance in the 1990s, because it was shown to retain higher efficacy than chloroquine against chloroquine-resistant parasites [5, 6]. Since then, amodiaquine has been used widely in Africa as an alternative to chloroquine or as second-line treatment. Today, amodiaquine is one of the most important front-line drugs for malaria control in Africa. In combination with artesunate, amodiaquine is used as first-line treatment in 17 countries [7]. In Kenya, amodiaquine was the second-line drug when sulfadoxine-pyrimethamine (SP) was the first-line drug per the national policy (1998–2004) [8] and was used as the first-line drug during the interim (2004–2006) before the implementation of artemether-lumefantrine as first-line treatment [9].

Although recent data reported by the East African Network for Monitoring Antimalarial Treatment (obtained using day 14 cure rates as the primary outcome measure) seem to support the continued use of amodiaquine [10], other in vivo studies from areas in both
West and East Africa, including Kenya, have reported increasing parasite resistance to amodiaquine [11–13]. There appears to be considerable heterogeneity in the geographical distribution of parasite sensitivity to amodiaquine. For instance, polymerase chain reaction (PCR)–corrected per-protocol treatment failure rates by day 28 in the amodiaquine monotherapy arm of a multicenter trial comparing amodiaquine with artesunate-amodiaquine for the treatment of uncomplicated P. falciparum malaria varied from 21% in Senegal and Gabon to an alarming 71% in Entasopia in southern Kenya [14] (M. Loolpapit, unpublished data).

The scarcity of recent in vitro sensitivity data from the East African region limits a comprehensive interpretation of the findings of in vivo studies, especially of those reported by effectiveness trials. One study from Rwanda showed a low prevalence (7%) of in vitro resistance to the major biologically active metabolite of amodiaquine, N-desethyl-amodiaquine (DEAQ) [15]. Comparative chemosensitivity data are also required for the appraisal of new amodiaquine derivatives as they progress through clinical development [16]. The aim of the present study was to determine the current pattern of in vivo and in vitro P. falciparum sensitivity to amodiaquine in an area in which the drug has been extensively used since 2003.

**METHODS**

**Study area.** The study was conducted at the Pingilikani Clinical Trials facility of the Kenya Medical Research Institute (KEMRI) Center for Geographic Medicine Research–Coast (CGMR-C), which is located 20 km south of the town of Kilifi, on the Kenyan coast. CGMR-C operates this study site adjacent to a primary health care clinic in collaboration with local Ministry of Health authorities. Pingilikani is in the southern part of the area that is demographically surveyed by the KEMRI center and is characterized by high year-round transmission of P. falciparum and an estimated entomological inoculation rate of between 22 and 53 infective bites per person per year [17], although the characteristics of transmission in the area are changing [18] (authors’ unpublished data). Subsidized insecticide-treated bed nets for children <5 years old and pregnant women are available at the Pingilikani Dispensary, and use is encouraged. The study was conducted during an interim period between a change of policy from sulfadoxine-pyrimethamine to artemether-lumefantrine as the first-line treatment for uncomplicated P. falciparum malaria. Amodiaquine has been used as first-line treatment for pediatric malaria case management at the Pingilikani Dispensary since 2003. The study protocol was reviewed and approved by the KEMRI Scientific Steering Committee and the National Ethical Review Committee.

**Study design and sample size.** The study was designed as a prospective observation of pediatric outpatients attending the Pingilikani Dispensary. Eligible patients received a supervised oral course of amodiaquine targeting a dose of 10 mg/kg/day for 3 days and were subsequently followed up for 28 days. Sample size estimation was based on the accepted practice of using a day 28 PCR-corrected cure rate of 90% (10% failure rate) as the cutoff point for differentiating between effective and failing drug therapies [19]. Our study was designed to detect parasitological failure rates of >10% by a ≧8% margin with 95% confidence, and, allowing for an estimated lost-to-follow-up rate of 17%, the minimum required sample size was calculated to be 120 patients.

**Enrollment of patients.** The study was conducted from March through July 2006. Pediatric patients attending the Pingilikani Dispensary were enrolled into the study if they met the following inclusion criteria: a history of fever during the preceding 24 h or an axillary temperature of ≧37.5°C; age of 6 months to 10 years; weight of ≧5 kg; microscopically confirmed P. falciparum monoinfection with an asexual parasite density of 2000–200,000 parasites/μL; resident in the study area; and provision of written informed consent by their accompanying parent or guardian. Exclusion criteria were as follows: adequate antimalarial intake for the current febrile illness; known hypersensitivity to amodiaquine; symptoms and signs of severe malaria [20]; any other severe underlying disease; concomitant disease masking the assessment of treatment response; danger signs; and severe malnutrition (weight-for-height values <70% of the median given on the National Center for Health Statistics/World Health Organization [WHO] reference tables).

**Study drug and administration.** Amodiaquine was formulated as 200-mg base tablets (Parke Davis), and the tablets (rounded to the nearest half tablet) were administered orally under direct supervision. Tablets were administered with drinking water; for infants and young children (<2 years old), the tablets were crushed, mixed with 25 mL of drinking water, and administered as slurry. Participants who vomited or rejected the study drug within 30 min received a second full dose, and those who vomited or rejected the study drug after 30 min but within 1 h received a second half dose. Vomiting or rejecting the second dose led to withdrawal from the study and administration of rescue medication (6 doses of oral artemether-lumefantrine over 3 days).

**Study flow and procedures.** Patients were seen by a study clinician for baseline assessment before administration of the first dose of study medication on days 0, 1, and 2 and again on days 3, 7, and 28 (or otherwise if indicated). Patients who failed to return for scheduled appointments were actively followed up in the community. On each visit during the treatment and follow-up phase, a medical history was obtained, vital signs were checked, axillary temperature was measured, thick and thin blood smears were prepared from a fingerprick blood sample, and adverse events were documented. Venipunctures were performed on days 0, 7, and 28 to monitor hemoglobin levels, hematocrit, and leukocyte counts (differential and platelet) as well as liver damage and renal function parameters (serum alanine...
aminotransferase and serum creatinine concentrations, respectively). To distinguish recrudescences from new infections, aliquots of ethylenediaminetetraacetic acid–treated blood samples obtained on day 0 and on the day of reappearing asexual parasitemia were stored at −80°C for PCR-based genotyping analysis. In addition, blood samples were collected in Vacutainer tubes (BD) containing acid citrate dextrose and transport medium for adaptation of *P. falciparum* in culture on day 0 and the day of parasite reappearance for in vitro drug-sensitivity assays.

**End points.** To measure the prevalence of in vivo parasite resistance, we defined the primary end point as the PCR-corrected parasitological cure rate on day 28 (“adequate clinical and parasitological response” as defined by the WHO [21]). Cure was defined as initial clearance of asexual parasites by day 7 and subsequent sustained absence of microscopically detected asexual parasitemia through day 28. Secondary end points included the rate of recurrences (including reinfections) on day 28; fever and parasite clearance times; the difference in in vitro chemosensitivity profiles between parasite isolates obtained from patients with and those without subsequent asexual parasite recrudescence and between parasite isolates obtained before treatment with amodiaquine and at the time of asexual parasite recurrence; mean change in hemoglobin concentration from day 0 to 28; and the incidence of clinical and laboratory-determined adverse events.

**Laboratory procedures.** Dried thick and thin blood smears were stained with 20% Giemsa solution (pH 7.2). Parasite density was determined by enumerating the number of parasites per 200 white blood cells, assuming a total white blood cell count of 8000 cells/µL. For safety evaluation, differential blood counts were measured using a semiautomated analyzer (AcT 5 Diff CP; Beckman Coulter), and clinical chemical analysis was done using a wet chemistry analyzer (Selectra E; Vitalab). To distinguish recrudescence from new infection, matched pairs of parasite isolates obtained on study admission and on the day of reappearing asexual parasitemia were compared using PCR-based genotyping analysis of repeat length polymorphisms in the *pfmsp2* gene locus [22]. We classified a recurrent asexual parasite infection after initial clearance as reinfection if the sizes of all electrophoretically separated PCR product bands detected for the day of reappearing asexual parasitemia were distinct from those detected for the day of study admission in at least 2 independent experiments.

**Parasite adaptation and chemosensitivity testing.** One milliliter of patient venous blood was collected and added to 4 mL of transport medium (Roswell Park Memorial Institute [RPMI] 1640 containing 10% albumin). Parasite suspensions were washed with normal RPMI 1640 and then cultured according to standard protocols (http://www.mr4.org). Parasite growth was monitored by light microscopy until a per-cycle increase in parasite...
load of >2-fold was observed, at which point the culture was diluted 2-fold. An isolate was considered to be adapted at a stable 2-fold per-cycle growth rate. The chemosensitivity of culture-adapted parasites to amodiaquine dihydrochloride dihydrate (AQ; Sigma), to the principal active circulating human metabolite DEAQ (LGC), and to chloroquine diphosphate (CQ; Sigma) was determined by the radioisotope-incorporation method in duplicate experiments [23]. Briefly, the culture was diluted to 0.5% parasitemia and a final hematocrit of 1.5%. Two hundred microliters of blood-medium mixture was added to 96-well microtiter plates containing the drugs in duplicate 3-fold serial dilutions (CQ, AQ, and DEAQ in medium ranging from 540 to 0.75 nmol/L for AQ, from 580 to 0.8 ng/mL for DEAQ, and from 450 to 0.6 nmol/L for CQ) prepared from stock solutions in 70% ethanol (for CQ and AQ) and 90% methanol plus 10% HCl (for DEAQ). These were incubated at 37°C in a gas mixture of 90% N₂, 5% O₂, and 5% CO₂ for 66 h. Growth was measured by determining the incorporation of [³H]- hypoxanthine, which was added during the last 18 h of incubation. Cells were lysed by freeze-thaw cycling of the plates. Cultures were harvested on fiberglass paper (PerkinElmer). Dried papers were immersed in scintillation fluid (PerkinElmer) before the amount of ionizing radiation was determined (1450 Microbeta Counter; Wallac). Results were expressed as the drug concentration required for 50% inhibition of [³H]-hypoxanthine incorporation into parasite nucleic acid compared with drug-free control (50% inhibitory concentration [IC₅₀]), using regression analysis of the dose–response curve.

Data management and statistical analysis. Data were captured using specifically designed source documents and were subsequently double-entered into an electronic database (FileMaker Pro; version 5; FileMaker). A commercial software package (Stata/MP; version 10; StataCorp) was used for data analysis. Recrudescence-free estimates were calculated as Kaplan-Meier product limits. Patients with genotypically confirmed new infections and patients who withdrew or were lost to follow-up were right censored. Times to asexual parasite and fever clearance were calculated from the start of treatment to the first of 2 consecutive parasite-free blood smears and axillary temperature measurements <37.5°C, respectively, and were derived from Kaplan-Meier analyses. Mean drug concentrations at 50% inhibition of growth were calculated by fitting a polynomial regression model. Regression models with $R^2$ values <0.9 were not included in analyses (Microsoft Excel 2008).

RESULTS

Study cohort. A total of 128 patients were enrolled into the present study and received at least 1 dose of amodiaquine. The baseline demographic and clinical characteristics of the patients enrolled in this study are summarized in table 1. Seventy-three percent (94/128) of the patients were <5 year old, and 93% (119/128) had been ill for ≤3 days. Baseline hematological and clinical chemistry parameters were within the reference range for the community with the exception of platelet counts, which were below the lower limit of the reference range in 57% (72/126) of patients. Figure 1 details the flow of patients through the study. A total of 110 patients reached a primary efficacy end point.

In vivo response. Results for the clinical and parasitological responses to treatment with amodiaquine are summarized in Figure 1.

![Figure 1. Study profile.](https://academic.oup.com/jid/article-abstract/199/11/1575/893270)

Table 2. Kaplan-Meier estimates of the in vivo responses to amodiaquine in pediatric outpatients with uncomplicated malaria.

<table>
<thead>
<tr>
<th>Treatment response</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean time to fever clearance, h</td>
<td>28 (26–30)</td>
</tr>
<tr>
<td>Mean time to asexual parasite clearance, h</td>
<td>67 (65–69)</td>
</tr>
<tr>
<td>Estimate for no recurrent infection on day 28, %</td>
<td>72 (62–79)</td>
</tr>
<tr>
<td>Estimate for recrudescent infection on day 28,a %</td>
<td>82 (74–88)</td>
</tr>
<tr>
<td>Estimate for reinfection on day 28, %</td>
<td>17 (11–26)</td>
</tr>
</tbody>
</table>

**NOTE.** Data in parentheses are 95% confidence intervals.

*a* Cure rate.
Two parasitologically defined failures occurred on day 2 (asexual parasite density higher than baseline). All remaining patients were free of parasites by day 7. Between days 7 and 28, we detected the recurrence of asexual parasite infection in 29 patients. Genotyping analysis revealed that 12 recurrent infections were reinfections, 10 were recrudescences, and 5 were mixed (recurrent isolates containing persistent and new alleles), which were classified as recrudescences for the primary endpoint analysis. Two cases for which PCR results were indeterminate were classified as recrudescences. Day 28 estimates are given in Table 2.

**In vitro response.** We first established the activity of DEAQ and AQ against reference laboratory strains V1/S [24] and 3D7 as resistant and sensitive controls, respectively. The AQ and DEAQ IC₅₀ values for V1/S were 15 and 97 nmol/L, respectively, and the AQ and DEAQ IC₅₀ values for 3D7 were 8 and 25 nmol/L, respectively, in agreement with the findings of previous studies using the same laboratory strains.

A total of 66 isolates (66/128 [46%]) obtained before treatment were tested for sensitivity to AQ and DEAQ, and a total of 19 isolates (19/29 [67%]) obtained from those with recurrent infections were tested. Of the 66 tested baseline isolates, 2 (3%) were resistant to amodiaquine but 17 (26%) were resistant to DEAQ when previously proposed arbitrary cut-offs of IC₅₀ values of >30 nmol/L [6] and >60 nmol/L [25], respectively, were applied. The median AQ IC₅₀ and DEAQ IC₅₀ values for isolates obtained before treatment from patients with versus those without subsequent asexual parasite recrudescence were similar (P > .3 for both comparisons): 16 nmol/L (95% CI, 7–46 nmol/L) and 29 nmol/L (95% CI, 23–170 nmol/L) versus 16 nmol/L (95% CI, 13–19 nmol/L) and 34 nmol/L (95% CI, 30–46 nmol/L), respectively (DEAQ IC₅₀ results are shown in Figure 3). The median AQ IC₅₀ and DEAQ IC₅₀ values for isolates obtained at baseline versus at asexual parasite recurrence were also similar (P > .5, for both comparisons): 16 nmol/L (95% CI, 13–19 nmol/L) and 33 nmol/L (95% CI, 30–43 nmol/L) versus 13 nmol/L (95% CI, 9–20 nmol/L) and 41 nmol/L (95% CI, 27–71 nmol/L), respectively (DEAQ IC₅₀ results are shown in Figure 3). Similarly, AQ IC₅₀ values for baseline isolates were also highly correlated (Spearman’s ρ = 0.75; P < .001) (Figure 4). AQ IC₅₀ values for baseline isolates were highly correlated with CQ IC₅₀ values (Spearman’s ρ = 0.53; P < .001).

**Safety and tolerability.** No serious adverse events were reported. A total of 74 clinical adverse events were observed after treatment. Adverse events commonly involved the gastrointestinal tract, the respiratory system, and the skin. Sixty-five (88%) of the adverse events were judged to be of mild intensity and 9 (12%) of moderate intensity, but none was judged to likely be related to amodiaquine intake. Most of the adverse events were also symptoms of malaria and, therefore, were likely to be related to disease. Changes in laboratory values after treatment were consistent with acute malaria and its resolution and are summarized in Table 3. Absolute neutrophil count decreased from day 0 to day 7 and then further dropped by day 28. A decline to National Institute of Allergy and Infectious Diseases–defined grade 1 neutropenia (white blood cell count of <1200 cells/μL) [26] after treatment was observed in 3 (3%) of 96 patients.

**DISCUSSION**

*P. falciparum* resistance to commonly used antimalarial drugs is a dynamic and important problem for public health, one that requires frequent and systematic monitoring of the efficacy of
drugs in vivo and/or testing parasite sensitivity in vitro. Amodiaquine is one of the most important drugs for malaria control in Africa, presently being used in combination with artesunate as first-line treatment or alone as second-line treatment in 17 African countries [7].

Parasite resistance to amodiaquine and its major active metabolite, DEAQ, remains a concern. Various levels of in vivo resistance to amodiaquine have been reported in East Africa, with day 14 failure rates ranging from an alarming 42% in Muheme, Tanzania [12], to <6% across national sentinel sites in Uganda [10]. In our study, the initial clinical and parasitological response rates to treatment with amodiaquine were satisfactory—with the notable exception of 2 parasitologically defined early treatment failures occurring on day 2—but we observed a high rate (18%) of late recrudescences on day 28 (Kaplan-Meier estimate). This rate is >3 times higher than the recommended 5% cutoff for an efficacious antimalarial drug [19] and, thus, cautions against the continued use of amodiaquine as a cheap and widely available second-line drug. The use of a single genetic marker to distinguish between recrudescence and reinfection in the present study can be considered a minor limitation, one that may have resulted in a slightly inflated estimate of the failure rate.

On oral administration, amodiaquine is rapidly and extensively converted during the first pass in the liver to DEAQ, the main pharmacologically active metabolite [27–29]. Although amodiaquine has higher intrinsic in vitro activity against chloroquine-sensitive isolates than DEAQ [30], the in vivo efficacy of amodiaquine is almost entirely due to DEAQ [31–33]. We detected parasite resistance to amodiaquine in only 2 (3%) of the 66 tested isolates, whereas 17 (26%) of the isolates were resistant to DEAQ when previously proposed arbitrary cutoffs of IC<sub>50</sub> values of >30 nmol/L [6] and >60 nmol/L [25], respectively, were applied. Our findings are in agreement with previous observations of near 100% prevalence of amodiaquine sensitivity among <i>P. falciparum</i> isolates obtained from African patients [34, 35], despite comparatively high rates of in vivo treatment failures [14, 15] and of in vitro resistance to DEAQ [15, 36]. This conflicting pattern could be explained by the rapid conversion of amodiaquine to DEAQ [32], resulting in minimal selective pressure on amodiaquine. The 2 early treatment failures in our study can therefore be interpreted as a result of (1) limited exposure to the highly active but quickly eliminated prodrug amodiaquine and (2) substantial parasite resistance to the main antimalarial metabolite of amodiaquine, DEAQ. On the other hand, the observed high correlation between in vitro responses to AQ and to DEAQ [34, 35], despite comparatively high rates of in vivo treatment failures [14, 15] and of in vitro resistance to DEAQ [15, 36].

Cutoff values have been proposed that would categorize in vitro responses of <i>P. falciparum</i> isolates as either sensitive or...
resistant to inhibitors of parasite growth [39] on the basis of the observation that baseline in vitro phenotypes are correlated with the outcome of antimalarial treatment (i.e., the likelihood of a treated asexual blood stage infection to recrudesce). However, this relationship, especially for intermediate in vitro inhibitory phenotypes, is nonlinear and is likely confounded by other key determinants of treatment outcome (e.g., immune status of the patient, baseline parasite biomass, and pharmacokinetic variables) [40, 41]. In the present study, a relatively high proportion of baseline isolates (29%) had DEAQ IC₅₀ values above the proposed cutoff of 60 nmol/L [25]; nonetheless, we failed to detect an association between baseline in vitro responses and subsequent risk of recrudescence.

In this study, we did not detect phenotypic signatures of selection for parasite resistance to amodiaquine or its major active metabolite, DEAQ, in field isolates after a single oral treatment course (43 nmol/L at recurrence vs. 33 nmol/L at baseline; \( P = .6 \)). This may be due to the small number of isolates obtained from patients with asexual parasite recurrence. Whether selection for DEAQ-resistant parasites is amplified by repeated exposure of incompletely eliminated blood stage infections can be investigated only in longitudinal studies of the effects of repeated treatment with the same drug for sequential malaria episodes [42]. We did not determine the IC₅₀ value for dihydroartemisinin (DHA). Such information may be valuable for examining the pattern of cross-resistance between DEAQ and DHA [43] and, thus, predicting the therapeutic life span of amodiaquine in combination with artemunate.

No serious adverse events were reported in our study. We observed an unexpected further drop in the median absolute neutrophil count after day 7. This contrasts with the typical pattern observed after antimalarial treatment [44, 45], in which an initial drop in absolute neutrophil count is followed by a subsequent recovery to normal values. In view of previous findings of potential bone marrow toxicity of amodiaquine when used for antimalarial chemotherapy [14, 46]—possibly mediated by bioactivation of amodiaquine to chemically reactive and cytotoxic intermediates [47] and/or induction of antibiotic antibodies [48]—our results reemphasize the need for long-term safety monitoring in countries in which amodiaquine-артемизин is used as first- or second-line treatment.

In summary, our findings of an 18% failure rate in vivo and of a 29% rate of resistant responses to DEAQ in vitro indicates the presence of a considerable level of amodiaquine resistance in Kilifi District, on the Kenyan coast. This result warrants continued attention to the value of amodiaquine combinations as first- or second-line treatment in areas with chloroquine-resistant parasites.

Acknowledgments

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References