IN VITRO ANTIMALARIAL SUSCEPTIBILITY PROFILE AND PRCRT/PFMDR-1 GENOTYPES OF PLASMODIUM FALCIPARUM FIELD ISOLATES FROM MALAWI

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Abstract. We measured in vitro antimalarial drug susceptibility of 84 Plasmodium falciparum field isolates from Blantyre, Southern Malawi, using the WHO micromethod and the lactate dehydrogenase assay. We also genotyped these isolates to investigate whether variation in their absolute drug sensitivity is associated with specific sets of pfcr and pfmdr-1 mutations harbored by parasites. Our results show that nearly a decade after the withdrawal of chloroquine (CQ) as a first-line antimalarial in Malawi, most isolates are now sensitive to CQ and none is CQ-resistant as predicted by their drug sensitivity phenotype and pfcr genotype. We also found that these isolates are uniformly sensitive to a range of quinoline-based antimalarials and artemisinin derivatives. These findings reinforce previous reports about a reduction in the proportion of CQ-resistant parasites after the withdrawal of CQ in 1993 and pave the way for re-assessing the clinical usefulness of CQ, artemisinins and other quinoline-based antimalarials in Malawi.

INTRODUCTION

The emergence and spread of resistance to front-line antimalarials are of huge public concern, and this has emphasized the need to continually field-monitor parasite susceptibility to conventional antimalarial drugs. Routine evaluation of the drug susceptibility of parasites will help to predict changes in baseline drug sensitivity over time and place to provide an early warning for emerging resistance or predict a reversion to sensitivity by the once-resistant parasite populations. Routine testing for antimalarial drug susceptibility will also aid the development of optimal malaria treatment policies.

In vivo and in vitro tests are the traditional tools used for evaluating antimalarial drug susceptibility in the field. The former, considered the “gold standard” for measuring antimalarial drug susceptibility, determines both the clinical condition of a patient after treatment with the antimalarial drug and the parasitological response to therapy. Several limitations of in vivo tests are recognized. They are operationally expensive to perform because of their demands on time, especially with protocols of 28 days or longer. In vitro tests may fail to discriminate between the true cases of drug resistance from treatment failures caused by factors other than parasite resistance to the drug, for example, non-compliance and pharmacokinetic variations. In addition, results from in vivo tests are often influenced by the immune status of the patient and ongoing malaria transmission. In contrast, in vitro tests measure intrinsic drug-sensitivity phenotype of parasites independently of the host immune status; unlike in vivo tests, pharmacokinetic variations do not influence test outcomes. However, in vitro tests have their own limitations. For example, parasite isolates from patients who have been on undisclosed self-treatment with antimalarials or antibiotics may not grow well in culture, and the interpretation of test results for polyclonal infections may be ambiguous. Another problem with in vitro tests is the determination of threshold IC50 values that distinguish the drug-susceptible parasites from the resistant ones. Currently, there are only a few validated cut-off points for assessing in vitro drug resistance. Despite these limitations, in vitro drug sensitivity tests are still of great value as they can be used to provide information on the baseline sensitivity of parasites to drugs in current use or to those that are about to be deployed or were once withdrawn from clinical use.

Several in vitro tests have been developed to measure the drug-susceptibility profile of malaria parasites in the field. These include (1) tests based on measuring the inhibitory effect of antimalarial drugs on parasite growth and development inside erythrocytes, such as the WHO micromethod; (2) assays based on assessing how much an antimalarial drug inhibits parasite uptake of radiolabeled growth precursors, for example, the H-hypoxanthine uptake assay; and (3) assays based on measuring the inhibition of enzymatic activity by an antimalarial drug, such as the lactate dehydrogenase (LDH) assay.

In this study, we evaluated the susceptibility of Plasmodium falciparum field isolates to a range of antimalarial drugs with a potential for deployment in Malawi using the WHO micromethod and the LDH assay. We then genotyped these isolates to determine whether specific sets of point mutations in pfcr and pfmdr-1 genes, previously associated with quinoline resistance, are associated with the level of parasite response to these drugs.

MATERIALS AND METHODS

Parasite isolates. P. falciparum isolates were collected from children < 5 years old presenting to Queen Elizabeth Central Hospital in Blantyre, Southern Malawi, between June 2003 and August 2004 and satisfying the following criteria: confirmed mono-infection with P. falciparum malaria (parasitemia between 1000 and 80,000 parasites per μL of blood), hematocrit ≥ 20% and no recent history of antimalarial or antibiotic use. Upon receiving consent from their parents or guardians, we collected venous blood samples (3–5 mL) from these children and stored them in sterile EDTA-coated Vacutainer tubes (BD Vacutainer Systems, Plymouth, U.K.). Samples were then transported in an ice-cold container to the laboratory where parasite cultivation, pfcr/pfmdr-1 genotyping and in vitro antimalarial susceptibility tests were performed. This study was part of a series of antimalarial drug...
resistance studies approved by the College of Medicine Research and Ethics Committee, University of Malawi.

**Parasite cultivation and maintenance.** *P. falciparum* isolates were grown and maintained in culture using a modification of the method of Trager and Jensen. Cultures consisted of a 1–6% suspension of O+ human erythrocytes in RPMI 1640 medium (Sigma-Aldrich Inc., St. Louis, MO) supplemented with gentamicin solution at 0.01 mg/mL, 25 mM HEPES buffer, 25 mM NaHCO₃, and 10% human AB serum from European donors without a history of malaria. Cultures were fed with a gas mixture of 4% O₂, 3% CO₂, and 97% N₂, and incubated at 37°C.

**Drug stocks.** Antimalarial test compounds were obtained from the following sources: amodiaquine hydrochloride (Sigma Chemical Co.), chloroquine diphosphate (Sigma Chemical Co.), mefloquine hydrochloride (Hoffman-La Roche, Basel, Switzerland), lumefantrine (Novartis Pharma AG, Basel, Switzerland), quinine hydrochloride (Sigma Chemical Co., arteether (Novartis Pharma AG), desethylamodiaquine (Sigma Chemical Co.), and dihydroartemisinin (Novartis Pharma AG). Stock solutions of amodiaquine, quinine, mefloquine, lumefantrine, arteether, desethylamodiaquine, and dihydroartemisinin were prepared in a 70% ethanol/water mixture. Those of chloroquine were prepared in sterile distilled water. All stock solutions were sterilized by passing them through a 20-µm filter and stored at −20°C until required.

**Determination of in vitro antimalarial drug susceptibility.** The susceptibility of isolates to various antimalarial drugs was measured using the WHO microtest assay and the LDH assay. Whenever the drug susceptibility of patient isolates was measured, a laboratory clone with known CQ-sensitivity status was included in the tests as a control. The WHO microtest was performed as previously described with slight modifications, and test results were interpreted as per protocol. LDH assays were performed in flat-bottomed 96-well microtiter plates as described by Makler and others (personal communication). Unparasitized O+ red blood cells without the drug were the blanks for the assay, and parasitized red blood cells without drug were the control for the assay. Parasites were plated in the trophozoite phase at 1% hematocrit and 2% parasitemia in 100 µL of an antimalarial drug at an appropriate, defined concentration. Plates were placed in a sealed jar and flushed with a gas mixture of 4% O₂, 3% CO₂, and 97% N₂, and incubated at 37°C for 48 hours. Meanwhile, two reagents for detecting and measuring the LDH enzyme were prepared. The first of these was the Malstat reagent, which was made by dissolving 400 µL of Triton X-100 in 80 mL of deionized water, adding 1-lactate (4.00 g), Tris buffer (1.32 g), and 0.022 g of 3-acetylpyridine adenine dinucleotide (APAD), adjusting the pH to 9 with hydrochloric acid, and bringing the volume up to 200 mL with deionized water. The second reagent is NBT/PES solution, and it was prepared by dissolving nitro blue tetrazolium salt (0.160 g) and phenazine ethosulfate (0.008 g) in 100 mL of deionized water. The solution was stored in a foil-covered container and kept in the refrigerator until required. All reagents for preparing the Malstat reagent and NBT/PES solution were purchased from Sigma-Aldrich Inc. (St. Louis, MO). When incubation was complete, plates were harvested and subjected to three 20-minute freeze–thaw cycles to resuspend the culture. Thereafter, 100 µL of Malstat reagent and 25 µL of NBT/PES solution were added to each well of a new, duplicate flat-bottomed 96-well microtiter plate. The culture in each of the wells of the original plate was resuspended by mixing with a multichannel pipette. Thereafter, 15 µL of the culture was taken from each well and added to the corresponding well of the Malstat plate, thereby initiating the lactate dehydrogenase reaction. Color development of the LDH plate was monitored colorimetrically at 620 nm with the aid of a plate reader after an hour of incubation in the dark.

**Analysis of test results from the LDH assay.** The LDH assay generates optical density (OD) values at various concentrations of the drug as raw data. OD values from control wells represent the maximum amount of LDH that is produced by parasites and OD values from blank wells represent background LDH activity. A 100% growth value, which corresponds to maximum LDH activity, was obtained by subtracting the mean OD value of blank wells from that of control wells. Likewise, the growth value at each concentration of the drug was obtained by adjusting OD values from drug-treated wells for background LDH activity. These values were then expressed as a percentage of the 100% growth value and plotted against corresponding concentrations of the drug using Graphit Software (Erithacus Software Ltd, Surrey, England) to generate log dose–response curves from which IC₅₀ values were obtained.

**Pfcrt and pfmdr-1 genotype analysis.** Parasite DNA for the analysis of pfcrt and pfmdr-1 genotypes was extracted from venous blood samples using the QIAamp DNA Mini Kit (Qiagen Ltd, West Sussex, U.K.). DNA from each isolate was assessed for the presence of six pfcrt mutations and two pfmdr-1 mutations associated with *P. falciparum* resistance to chloroquine and other quinoline-based antimalarials. Point mutations that we looked for in pfcrt include K76T (substitution of threonine for lysine), A220S (substitution of serine for alanine), Q271E (substitution of glutamic acid for glutamine), N326S (substitution of serine for asparagine), I356T (substitution of threonine for isoleucine), and R371I (substitution of isoleucine for arginine). The pfmdr-1 mutations that we looked for are N86Y (substitution of tyrosine for asparagine) and D1246Y (substitution of tyrosine for aspartic acid). Nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) assays were used to detect the presence of these point mutations as previously described.

**Statistical analysis of data.** IC₅₀ values were log-transformed and expressed as the geometric mean IC₅₀ and 95% confidence intervals for the geometric mean were calculated. Correlations between IC₅₀ values of antimalarial drugs were measured by the Pearson’s correlation coefficient (r), and the potential for cross-resistance between any two drugs was assessed by standard linear regression analysis. Student’s t test for independent samples was used to assess whether the geometric mean IC₅₀ values of drugs were significantly different from each other. For all statistical tests, the level of significance (P) was set at 5%. Binomial exact 95% confidence intervals for the prevalence of the K76T pfcrt mutation were derived using STATA version 8.1 (Stata Corporation, College Station, TX).

**RESULTS**

**In vitro CQ sensitivity and pfcrt/pfmdr-1 genotypes of the first 56 isolates.** Fifty-six of 61 patient isolates were success-
fully assayed for in vitro CQ susceptibility using the WHO microtest assay. Of these, 41 (73%) were determined to be fully sensitive to CQ and the rest (27%) were of intermediate CQ susceptibility (Table 1). Some isolates were highly sensitive to CQ, as indicated by lack of schizont growth even at 0.2 μmol of CQ per liter of blood. None of these isolates was fully resistant to CQ as measured by its drug-sensitivity phenotype. The key CQ-resistance–conferring mutation at codon 76 of the pfcrt gene (K76T mutation) was not found in any of these isolates. Analysis of other polymorphic sites in pfcrt revealed the absence of CQR-associated mutations at codons 220, 271, 326, 356, and 371 in these isolates (Table 1). However, two isolates with a CQ-sensitive phenotype carried a mixture of wild-type and mutant pfmdr-1 alleles at codon 1246 and another CQ-sensitive isolate was found to harbor a mutant pfmdr-1 allele at position 86.

In vitro sensitivity of 28 patient isolates to diverse anti-malarial agents. We used the LDH assay to determine the susceptibility of patient isolates to various anti-malarial drugs with a potential for use in Malawi. Of 28 patient isolates subjected to in vitro susceptibility testing, 21 had their responses to chloroquine (CQ), amodiaquine (AQ), desethylamodiaquine (dAQ), quinine (QN), mefloquine (MQ), dihydroartemisinin (DHA), artemether (ART), and lumefantrine (LM) fully characterized. Table 2 summarizes in vitro responses of these isolates to a range of anti-malarial drugs. The successfully assayed isolates had a geometric mean CQ IC50 of 26.4 nM; range 15–63 nM. The activity of AQ against these isolates was slightly higher (geometric mean IC50 = 23.0 nM; range 12–40 nM) but not significantly different (P = 0.091) from that of CQ. The activity of dAQ (geometric mean IC50 = 64.1 nM; range 40–94 nM) against these isolates was significantly lower (P < 0.001) compared with that of CQ and AQ. The activity of dAQ against most individual isolates was ≈2–3 times lower than that of CQ and AQ (data not shown). The geometric mean CQ values of these isolates for other drugs were as follows: MQ (geometric mean IC50 = 24.1 nM; range 13–31 nM), QN (geometric mean IC50 = 146.0 nM; range 100–246 nM), DHA (geometric mean IC50 = 91.9 nM; range 5–15 nM), ART (geometric mean IC50 = 15.1 nM; range 7–25 nM), LM (geometric mean IC50 = 90.1 nM; range 48–129 nM). Artemisinin compounds were the most potent anti-malarials against Malawian isolates and the order of activity of various drugs against these isolates was: DHA < ART < (CQ, AQ, MQ) < dAQ < LM < QN. Subsequent analysis of parasite DNA from these isolates revealed that two isolates had the 86Y pfmdr-1 mutation, and none carried any of the pfcrt mutations implicated in chloroquine resistance. The 86Y pfmdr-1 mutation appeared to be associated with enhanced parasite sensitivity to both MQ and LM but with reduced sensitivity to CQ.

Relationships between in vitro activities of anti-malarial drugs. Figure 1 shows scatter plots depicting the relationship between in vitro activities of anti-malarial drugs. The scatter plot of log CQ IC50 values against those of AQ showed that the activities of these compounds were strongly and significantly associated (r = 0.628, P = 0.001). The in vitro activity of CQ was also strongly and significantly associated with that of dAQ (r = 0.621, P = 0.001). The correlation between the activity of AQ and that of its active metabolite, dAQ, was also strong and significant (r = 0.756, P < 0.001). Quinine IC50 values of patient isolates were moderately and significantly correlated with those of CQ (r = 0.457, P = 0.019). Artemether IC50 values were weakly but positively associated (r = 0.293, P = 0.197) with those of DHA. Another strong and significant association was observed between the in vitro activities of MQ and LM (r = 0.777, P < 0.001). In contrast, IC50 values of MQ, DHA, ART, and LM were weakly and negatively associated with those of CQ (data not shown).

Table 1: In vitro chloroquine sensitivity and pfcrt/pfmdr-1 genotypes of 56 field isolates

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>MIC*</th>
<th>Sensitivity†</th>
<th>pfcrt</th>
<th>pfmdr-1*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>76</td>
<td>220</td>
</tr>
<tr>
<td>2</td>
<td>&lt; 0.20</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>0.40</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>0.40</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>0.40</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>0.80</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>23</td>
<td>≤ 0.80</td>
<td>S</td>
<td>K</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>≤ 1.60</td>
<td>IS</td>
<td>K</td>
<td>A</td>
</tr>
</tbody>
</table>

* MIC refers to the minimum inhibitory concentration of chloroquine that arrests the development of ring-stage parasites into schizonts and is reported in μM.
† The chloroquine sensitivity status of isolates is reported as S for "sensitive" and IS for "intermediate sensitivity."
‡ Numbers 76–1246 refer to amino acid positions in PCRT and PGH-1; letters are abbreviations for the different amino acids (for example, N = asparagine).

Table 2: In vitro susceptibility of 28 patient isolates to various anti-malarial agents

<table>
<thead>
<tr>
<th>Drug</th>
<th>No.</th>
<th>GM IC50 (nM)</th>
<th>95% CI for GM IC50 (nM)</th>
<th>Trt (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>27</td>
<td>26.4</td>
<td>23.3–29.9</td>
<td>100</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>27</td>
<td>23.0</td>
<td>20.9–25.4</td>
<td>80</td>
</tr>
<tr>
<td>Desethylamodiaquine</td>
<td>28</td>
<td>64.1</td>
<td>58.5–70.2</td>
<td>60</td>
</tr>
<tr>
<td>Quinine</td>
<td>27</td>
<td>146.0</td>
<td>132.0–161.5</td>
<td>450</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>21</td>
<td>24.1</td>
<td>22.2–26.0</td>
<td>30</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>21</td>
<td>9.1</td>
<td>7.9–10.5</td>
<td>NE</td>
</tr>
<tr>
<td>Artemether</td>
<td>21</td>
<td>15.1</td>
<td>13.6–16.8</td>
<td>NE</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>21</td>
<td>90.1</td>
<td>83.5–97.2</td>
<td>NE</td>
</tr>
</tbody>
</table>

* Drug sensitivity data are quoted as the geometric mean 50% inhibitory concentration (GM IC50) for all the successfully tested isolates; 95% CI for GM IC50 (nM) = 95% confidence intervals for the geometric mean 50% inhibitory concentration; n = number of isolates successfully assayed for in vitro anti-malarial drug susceptibility.
† Tr stands for threshold resistance and represents previously defined cut-off concentration that distinguishes drug-susceptible parasites from resistant ones. 50% NE = not established; IC50 values for the reference drugs: 3D7: chloroquine = 22 nM; amodiaquine = 15 nM; desethylamodiaquine = 26 nM; quinine = 127 nM; mefloquine = 25 nM; dihydroartemisinin = 9 nM; artemether = 13 nM; lumefantrine = 79 nM.

DISCUSSION

Sensitivity of patient isolates to diverse anti-malarial drugs. Results from this study indicate that Malawian isolates have a high degree of in vitro sensitivity to all the quinoline anti-malarials and artemisinin drugs that we studied. These drugs have not been heavily used in Malawi in recent times. Quinine is regularly prescribed but only for severe disease and SP treatment failures. Chloroquine (CQ) was comprehensively withdrawn from use as first-line therapy in 1993. Kublin and others reported that the prevalence of the K76T pfcrt mutation, associated with CQ resistance, declined progressively and significantly from 85% in 1992 to 13% by 2000. In the present study, we found that the prevalence of the K76T pfcrt mutation has fallen further to 0% (95% CI for prevalence: 0–4%). No case of in vitro chloroquine resistance
(IC$_{50} > 100$ nM) was found among isolates that we analyzed; this is consistent with the observation that all isolates had a wild-type pfcrf genotype, which is highly predictive of CQ sensitivity.$^6,^{13}$ These findings add further to the growing weight of evidence for the re-emergence of CQ-sensitive parasites in Malawi.$^{12,14,15}$ Complemented by the finding that asymptomatic infections are cleared by standard doses of CQ in semi-immune individuals from the same area,$^{12}$ these data have paved the way for evaluating the clinical utility of CQ in individuals with symptomatic malaria. Meanwhile, investigators working in the Ndirande Township located on the outskirts of Blantyre have reported a 28-day adequate clinical and parasitological response rate of 99% (95% CI: 93–100%) in 80 children treated with CQ for uncomplicated malaria.$^{17}$ This finding suggests a full return to CQ-sensitivity by the once CQ-resistant parasite population and raises the attractive possibility that CQ might once again become useful in the control of malaria in this setting. Amodiaquine (AQ), which

**Figure 1.** Scatter plots and regression lines showing the relationship between IC$_{50}$ values of different antimalarial drugs for the same patient isolates.
is very similar in structure to CQ and is under evaluation as a potential component of combination therapy in Malawi, was as active as CQ against parasite isolates in the present study. Although mefloquine (MQ) showed satisfactory activity against Malawian isolates (geometric mean IC_{90} = 24.1 nM), IC_{90} values of some isolates were not far off from the threshold of resistance (30 nM).\textsuperscript{7,9} This observation suggests that it might require a relatively short period of drug pressure to select for MQ resistance in Malawi. Nonetheless, MQ is unlikely to be a candidate for use by the national malaria control program because of its high cost. Its closely related drug, lumefantrine, which is increasingly being deployed in combination with artemether in the region, showed lower levels of antiparasitic activity in our study than in a similar study from Cameroon.\textsuperscript{18} This is cause for some concern because lumefantrine has a longer half-life than artemether in the human host, so that lumefantrine persists alone at subtherapeutic blood concentrations for some weeks after combination therapy and may exert selection pressure for resistant parasites. As in some previous studies,\textsuperscript{20,21} however, the potential for cross-resistance between MQ and CQ observed in the present study was not evident in other studies.\textsuperscript{22–24} The basis of these discrepancies remains unknown but they may in part be attributable to methodological differences in the assessment of in vitro antimalarial activity. Although in areas of high-level CQ resistance, AQ treatment failures are often thought to be due to cross-resistance between dAQ and CQ,\textsuperscript{22,23} results from this study suggest that AQ as a parent drug could also fail by virtue of its cross-resistance pattern to CQ. It is possible that cross-resistance between AQ and CQ (which is common in vivo) is governed by the degree of in vitro dealkylation of AQ to its active metabolite, dAQ. If AQ is extensively metabolized into dAQ, which is less potent perhaps because of its lower lipophilicity than AQ,\textsuperscript{25,26} there may be a greater degree of cross-resistance with CQ, which is less lipophilic than dAQ. Results from the present study also suggest that there is potential for cross-resistance between quinine (QN) and chloroquine (Figure 1). This finding is consistent with results from a series of drug-resistance studies conducted in East Africa, which showed cross-resistance between CQ and QN in some CQ-resistant isolates.\textsuperscript{27} However, in the present study, we could not find a strong relationship between the activities of CQ and other quinoline-based drugs, such as MQ and LM, which belong to the same family as QN. The activities of MQ and LM were negatively correlated to that of CQ (data not shown). The inverse relationship between the activities of MQ and CQ is well described\textsuperscript{26–28} and is thought to explain reports of reduced CQ resistance in areas where MQ resistance is increasing.\textsuperscript{34} However, in a comprehensive drug-susceptibility study of field isolates from sub-Saharan Africa,\textsuperscript{35} this inverse relationship was not borne out. In this study, we found the activities of MQ and LM to be closely associated (r = 0.777), and it is very unlikely that this correlation might have arisen by chance (P < 0.001). A mutation that results in the substitution of the amino acid tyrosine (Y) for asparagine (N) at codon 86 of pfmdr-1 appeared to enhance the activities of MQ and LM. This observation suggests that the inverse relationship, which exists between the in vitro activities of MQ and CQ, may be partly related to the structure of P-glycoprotein homologue-1, encoded by pfmdr-1.

In conclusion, results from this study highlight the high degree of susceptibility of local parasites in Malawi to a wide range of quinoline-based antimalarials and artemisinin-type drugs. Although parasite response to chloroquine was variable, no case of in vitro resistance was found. Parasite responses to other quinoline-based drugs and artemisinin derivatives showed a similar pattern to that of CQ, and no case of resistance was found among the successfully assayed isolates. These data indicate the potential clinical utility of quinoline-based antimalarials and artemisinin-type drugs in Malawi. In future, Malawi may be in a position to utilize some of these drugs in low-cost combination strategies.

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