LACK OF PREDICTION OF MEFLOQUINE AND MEFLOQUINE-ARTESUNATE TREATMENT OUTCOME BY MUTATIONS IN THE PLASMODIUM FALCIPARUM MULTIDRUG RESISTANCE 1 (Pfmdr1) GENE FOR F. FALCIPARUM MALARIA IN PERU

DYLAN R. PILLAI, GISELY HIJAR, YSABEL MONTOYA, WILMER MARQUIÑO, TRENTON K. RUEBUSH II, CHANSUDA WONGSrichanalai, AND KEVIN C. KAIN

Tropical Disease Unit, Toronto General Hospital and University of Toronto, Toronto, Ontario, Canada; Instituto Nacional de Salud, Lima, Peru; U.S. Naval Medical Research Center Detachment, Lima, Peru; Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Abstract. We assessed whether mutations in the Plasmodium falciparum multidrug-resistance gene 1 (pfmdr1) (C1034S, D1042N, and Y1246D) would predict treatment outcome during a 28-day in vivo treatment trial in the Peruvian Amazon. Mefloquine (MQ) was compared with mefloquine-артесunate (MQ-AS) in a randomized, multi-clinic protocol for the first time in the Americas. Of 115 patients enrolled in the in vivo arm, 97 patients were eligible for molecular analysis. All 97 patients remained parasite-free during 28 days of follow-up (MQ, n = 46; MQ-AS, n = 51), indicating 100% clinical efficacy of the MQ and MQ-AS treatment regimens. The reported MQ-sensitive alleles (C1034, D1042, and Y1246) were present in 48.5% (n = 47) of the cases, whereas 49 isolates (50.5%) contained the D1246 mutation reported to confer MQ resistance in vitro. However, neither this mutation nor a double mutation (S1034, D1246; n = 16) was predictive of MQ treatment outcome.

INTRODUCTION

Between 1992 and 1997, the incidence of Plasmodium falciparum malaria in the Peruvian Amazon region increased approximately 50-fold, due primarily, it is believed, to invasion of the region by the very efficient vector Anopheles darlingi.1 Peruvian public health authorities have traditionally relied on chloroquine and sulfadoxine-pyrimethamine as first-line therapy against P. falciparum malaria, but over the last decade, chloroquine-resistant and sulfadoxine-pyrimethamine-resistant P. falciparum malaria has come to predominate in the Peruvian Amazon.2 In an effort to assess the efficacy of alternative antimalarial drugs, the Peruvian Ministry of Health undertook a comparative trial of mefloquine (MQ) versus a combination of mefloquine-артесunate (MQ-AS) in Southeast Asia. 5 Resistance to MQ subsequently occurred over two decades ago in Southeast Asia.6 Resistance to MQ subsequently emerged along the Thai-Myanmar and Thai-Cambodian borders, and the National Malaria Control Program of Thailand responded by introducing the combination of MQ plus AS, which appears to have halted the steady increase in MQ resistance.7 Recent in vitro data relying on parasite transfection demonstrate that key point mutations in the P. falciparum multidrug resistance 1 (pfmdr1) gene, which encodes the transmembrane efflux protein Pgh1, confers resistance to MQ in vitro and a two-fold increase in the 50% inhibitory concentration (IC50) to AS.8 Reed and others have shown that the mutations C1034S, D1042N, and, in particular, Y1246D can increase the IC50 to both MQ and AS, suggesting a common mechanism of drug resistance.2 These experiments were performed in part using the MQ- and AS-resistant laboratory isolate 7G8, a South American strain, making this in vivo study particularly relevant. The purpose of this study was to 1) identify the baseline prevalence of pfmdr1 resistance mutations in this region of Peru, and 2) determine whether pfmdr1 mutations could serve as a useful molecular predictor of MQ versus MQ-AS treatment outcome.

MATERIALS AND METHODS

Ethical guidelines. Informed written consent was obtained from all human adult participants and from parents or legal guardians of minors. This study was approved by the Institutional Review Boards of the Centers for Disease Control and Prevention (Atlanta, GA), the U.S. Naval Medical Research Center (Forest Glen, MD), and the Instituto Nacional de Salud (Lima, Peru).

In vivo study. The 28-day World Health Organization in vivo treatment study was carried out in the city of Iquitos, Department of Loreto in the Peruvian Amazon during the peak transmission season of June through September 2000.9 Study sites were established at the Centro de Salud Moronococha and Hospital de Apoyo in Iquitos, both urban clinics visited by patients both within Iquitos city limits and from villages along tributaries of the Amazon. Symptomatic patients (axillary temperature >37.5°C and or a history of fever in the previous 48 hours) were selected for enrolment into the in vivo study arm based on the presence of >500 P. falciparum asexual parasites/μL on thick blood films. Subjects were randomized to either MQ (15 mg/kg, single dose) or MQ and AS (4 mg/kg/day over a three-day period) and followed over a 28-day period to document both clinical improvement and parasitologic clearance.9,10 Patients were excluded if they were less than five years old or more than 50 years old, pregnant, or demonstrated signs and symptoms suggestive of severe malaria.

Blood collection and purification of DNA. Blood was collected on day 0 by fingerprick and 500 μL was stored frozen in cryotubes containing EDTA (Nunc, Rochester, NY). Further blood samples were obtained on days 3, 7, 14, 21, and 28 and with any recurrence of symptoms suggestive of possible re-infection/recrudescence. Extraction of DNA from blood samples was performed using QIAamp spin columns (Qiagen, Valencia, CA) according to the manufacturer's
guidelines and stored in aliquots at -20°C until amplification by a polymerase chain reaction (PCR).

**Amplification by PCR and sequencing of DNA.** The DNA primers mdrl (5'-GCT ATT GAT TAT AAA AAT AAA GGA C-3') and mdr2 (5'-CCA AAT TTG ATA TTG TCA TAT ATG GAC-3') were designed to amplify a 747-base pair product (3539 to 4286 of pfmdr1; Genbank Accession number X56851) spanning positions 1034, 1042, and 1246. Amplification conditions were as follows: a hot start at 94°C for five minutes prior to adding Taq DNA polymerase, then 35 cycles of denaturation at 94°C for one minute, annealing at 50°C for one minute, and extension at 72°C for one minute. The PCR products (750 base pairs) were analyzed by agarose gel electrophoresis and then directly purified by MinElute PCR purification spin columns (Qiagen). Sequencing of DNA was performed according to standard methods.11

### RESULTS

One hundred fifteen patients were enrolled into the treatment study and randomized to receive either MQ or a combination of MQ-AS (Table 1). Eighteen patients were excluded from the molecular analysis due to insufficient parasitemia at day 0 (n = 4), loss to follow-up during the 28-day trial (n = 13), or difficulties with DNA purification and amplification (n = 1). The remaining 97 patients were parasitefree up to day 28 of follow-up, indicating 100% efficacy of MQ (n = 46) and MQ-AS (n = 51) combination therapy in this cohort. No significant demographic differences were present between each arm.

Sequencing analysis of the DNA of the pfmdr1 gene from isolates demonstrated that the 7G8-like, fully MQ-sensitive allele C1034, D1042, Y1246 was present in 48.5% (n = 47) of samples.7 Forty-nine isolates (50.5%) contained the D1246 mutation reported by Reed and others as sufficient to confer MQ resistance in vitro.2 However, this mutation was not predictive of MQ treatment outcome in this study. Interestingly, 16.5% (n = 16) of the isolates possessed a S1034, D1042, D1246 mixed allele and would require only one further mutation at residue 1042 to obtain the triply-mutated pfmdr1 allele also reported with MQ resistance (Figure 1).7

Since no in vivo resistance was documented in this cohort, MQ-resistant isolates MS98-08 (MQ IC50 = 70.18 ng/mL) and BR97-07 (MQ IC50 = 69.04 ng/mL) from patients in Thailand were used as controls. These isolates have IC50 values in the same range as the 7G8 single mutant (D1246) and triple mutant (S1034/N1042/D1246) generated by Reed and others in vitro.7 Sequencing of the DNA of the pfmdr1 gene from the Thai MQ-resistant isolates revealed the triple pfmdr1 resistant allele S1034, N1042, D1246 as would be predicted, whereas MQ-sensitive isolate 7G8 (MQ IC50 = 15.2 ng/mL) retained the fully sensitive allele C1034, D1042, Y1246 (Figure 1).

### DISCUSSION

This study represents the first randomized comparative efficacy trial of MQ and MQ-AS in the Americas and the results demonstrate that both treatment regimens are viable alternatives as first-line therapy in this region of Peru. This information is relevant in the context of rapidly emerging resistance to current first-line therapy with chloroquine and sulfadoxine-pyrimethamine, as well as reports of emerging resistance to MQ in neighboring Brazil.12 Molecular analysis of mutations linked with resistance to these anti-malarials provides important baseline data of pfmdr1 mutations present in this region. The majority of P. falciparum isolates evaluated in this study possessed sensitive or mixed pfmdr1 alleles. Reed and others7 have previously shown that the single pfmdr1 mutation Y1246D is sufficient to dramatically increase IC50s to MQ and, to a lesser degree, AS, in vitro, at least on the genetic background of 7G8, a South American isolate. In our cohort, more than 50% of the isolates contained this mutation, but no MQ treatment failures occurred, indicating that this mutation does not appear to be sufficient to confer MQ resistance in vivo. In previous reports using the P. falciparum chloroquine resistance transporter (pfcrt) gene as a molecular marker for chloroquine-resistant P. falciparum, chloroquine therapy cleared some infections in which parasites possessed the pfcrt chloroquine resistance allele T76, an effect attributed to premunition or partial immunity in highly endemic areas.13 Premunition seems a less likely explanation for our findings since in contrast to hyperendemic areas in sub-Saharan Africa, this study was carried out in a hypoendemic to mesoendemic region for malaria transmission, suggesting that acquired immunity is not likely to play a major role in clearance of resistant parasites. This is further shown by the mean age of our study participants (26.2 years old).

An alternative explanation may be that multiple mutations are required for an in vivo resistance phenotype.14 In this regard, 16.5% of the isolates examined were only one mutation shy of the triply-mutated pfmdr1 allele also linked to MQ resistance in vitro.2 Although this study was limited by the absence of observed in vivo resistance, in vivo MQ-resistant isolates from Southeast Asia possessed the N1042 mutation in pfmdr1. In future studies it will be of interest to undertake periodic molecular surveys of this sentinel residue to determine if it serves as a useful molecular predictor of emerging MQ resistance following the implementation of MQ and AS as first-line therapy in the Peruvian Amazon region. Future studies will also require analysis of pfmdr1 copy number since this may also influence resistance patterns, although conflicting data exist at present.

*No significant demographic differences were present between each arm of the study.
†MQ-AS = mefloquine and artesunate (4 mg/kg/day over a three-day period).
‡MQ-AS = mefloquine (15 mg/kg, single dose).

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Authors’ addresses: Dylan R. Pillai, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305. Gisely Hijar, Ysabel Montoya, and Wilmer Marquiño, Departments of Molecular Biology and Parasitology, Instituto Nacional de Salud, Lima, Peru. Trenton K. Ruebush II, Center for Disease Control and Prevention, Atlanta, GA. Chansuda Wongsrichanalai, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Kevin C.
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