Am. J. Trop. Med. Hyg., 81(1), 2009, pp. 13–18

Molecular Epidemiology of Malaria in Cameroon. XXVIII. *In vitro* Activity of Dihydroartemisinin against Clinical Isolates of *Plasmodium falciparum* and Sequence Analysis of the *P. falciparum* ATPase 6 Gene

Rachida Tahar, Pascal Ringwald, and Leonardo K. Basco*

Unité de Recherche Maladies Infectieuses et Tropicales Emergentes, Institut de Recherche pour le Développement and Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la Lutte Contre les Endémies en Afrique Centrale, Yaoundé, Cameroon; Antimalarial Drug Resistance, Global Malaria Programme, World Health Organization, Geneva, Switzerland

Abstract. The *Plasmodium falciparum* ATPase 6 (*Pfatp6*), homolog of sarco-endoplasmic reticulum, calcium-dependent ATPase in malaria parasites, has been proposed to be the main target of artemisinins. Four distinct point mutations (L263E, E431K, A623E, and S769N) have been reported to be associated with artemisinin resistance. The *Pfatp6* sequence polymorphism was determined to evaluate the prevalence of these mutations in fresh clinical isolates in Yaounde, Cameroon, and compare sequence data with *in vitro* response to dihydroartemisinin. Two major haplotypes were observed: the wild-type LEAS (n = 60, 62%) and a single mutant LKAS (n = 35, 36%). These amino acid substitutions did not influence the level of *in vitro* response to dihydroartemisinin ($P > 0.05$). *Plasmodium falciparum* isolates from Cameroon are highly sensitive *in vitro* to artemisinins. However, the relatively high prevalence of E431K may be a warning signal that warrants a regular monitoring of these molecular markers and/or *in vitro* activity of artemisinin derivatives.

INTRODUCTION

Artemisinins remain the main hope for current malaria control efforts because of their effectiveness for the treatment of multidrug-resistant *Plasmodium falciparum* infections and rapid action to treat severe and complicated malaria.[1] As recommended by the World Health Organization,[2] many countries where malaria is endemic, including those in Africa, have recently adopted, or are in the process of adopting, artemisinin-based combination therapy (ACTs) as the first-line drug for the treatment of uncomplicated malaria. This strategy improves antimalarial treatment effectiveness and is expected to delay the emergence of artemisinin-resistant parasites by rendering the probability of selecting drug-resistant parasites unlikely or very low. This assumption is based on the mutually protective action of artemisinin derivatives and other old and new antimalarial drugs, such as amodiaquine, sulfadoxine-pyrimethamine, mefloquine, lumefantrine, piperaquine, and pyronaridine.[3] Artemisinins rapidly decrease the parasite biomass in the human host, and their drug partner, with relatively slower action, eliminates residual parasites.

The endoperoxide bridge has been demonstrated to be the key structure necessary for the schizontocidal action of artemisinins; its derivative lacking this bridge is inactive.[4] The precise mechanism of action of artemisinins has not been elucidated. It has been suggested that a heme-dependent cleavage of endoperoxide bridge by iron-sulfur oxido-reduction within the food vacuole of the parasite and production of free radicals lead to alkylation and inhibition of functional parasite proteins.[5] Another hypothesis is based on the classic enzyme-antagonist model in which artemisinins are thought to specifically inhibit a calcium-dependent ATPase, the sarco-endoplasmic reticulum calcium-dependent ATPase (SERCA) ortholog of *P. falciparum* ATPase 6 (*Pfatp6*), outside the food vacuole.[6]

Until recently, there have been only two documented cases of clinical resistance to artemisinins.[7] Clinical failures to artemisinin monotherapies observed in the past have usually been ascribed to pharmacokinetic factors, in particular rapid elimination half-life and inadequate dosage.[8] However, laboratory studies have shown that *P. falciparum* can develop stable resistance to artemisinin with high inhibitory concentrations (50% inhibitory concentration [IC_{50}]), which suggest a potential for the emergence of *in vivo* drug resistance after a prolonged and extensive use of these drugs.[9] Two candidate proteins that may explain artemisinin resistance have emerged in recent years. Several studies have suggested the possible role of gene amplification of or specific mutations in the *P. falciparum* multidrug-resistant 1 gene (*pfmdr1*) in artemisinin resistance (also mefloquine, halofantrine, and lumefantrine resistance).[10–12] Other studies have not confirmed the association between *pfmdr1* and artemisinin (and amino alcohol) resistance.[13–16] An alternative basis of artemisinin resistance involving point mutation(s) in *pfatp6* has been recently suggested. Using *Xenopus laevis* oocyte expression system and enzymology methods, Uhlemann and others found that L263E substitution can overcome SERCA inhibition by artemisinins.[17]

Further *in vitro* studies that compared the low level of artemisinin sensitivity (artemether $IC_{50} > 30$ nM) in field isolates and *pfatp6* sequences have suggested three amino acid substitutions that may be associated with artemisinin resistance: E431K and A623E in West Africa and S769N in South America.[18] It remains to be established whether these mutations lead to clinical resistance to artemisinins and/or ACTs. The aim of this study was to analyze *Pfatp6* sequence polymorphisms in fresh clinical isolates collected from 2001 through 2006 in Yaoundé, Cameroon, and compare the data with dihydroartemisinin IC_{50}s.

PATIENTS AND METHODS

Blood collection. After informed consent was obtained, fresh clinical isolates of *P. falciparum* were collected by venipuncture (5–10 mL of blood in EDTA-coated collection tubes) from symptomatic patients ≥ 12 years of age who came to the Nlongkak Catholic missionary dispensary for

* Address correspondence to Leonardo K. Basco, Unité de Recherche Maladies Infectieuses et Tropicales Emergentes, Institut de Recherche pour le Développement and Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la Lutte Contre les Endémies en Afrique Centrale, BP 288, Yaoundé, Cameroon. E-mail: lkbasco@yahoo.fr

febrile episodes in Yaoundé in 2001, 2002, 2003, and 2006. The inclusion criteria were a parasitemia ≥ 0.1%, absence of other *Plasmodium* species, and denial of recent self-medication with an antimalarial drug confirmed by the Saker-Solomons urine test.[19] Children < 12 years of age, pregnant women, and patients with signs and symptoms of severe and complicated malaria were excluded. The enrolled patients were treated with either amodiaquine monotherapy or an amodiaquine–sulfadoxine-pyrimethamine combination in 2002–2003 and artesunate–amodiaquine in 2006. The study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

***In vitro* assay.** Artemisinin was obtained from the Sigma Chemical Co. (St. Louis, MO). Several batches of dihydroartemisinin were obtained from Sapec (Lugano, Switzerland) and Shin Poong Pharmaceutical Co. (Seoul, South Korea) through the courtesy of the World Health Organization (Geneva, Switzerland). Stock and working solutions were prepared in methanol and distributed in triplicate in 96-well culture plates and dried before use, as described in previous work.[20]

The *in vitro* response was determined by the isotopic microtest.[21] Blood samples were transported to our laboratory within 1–2 hours after collection. Infected erythrocytes were washed three times with RPMI 1640 medium buffered with 25 mM *N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) and 25 mM $NaHCO_3$ by centrifugation and suspended in the complete RPMI 1640 medium with 10% non-immune, type AB^+ human serum (batch pooled from donors at the Blood Transfusion Center, Strasbourg, France for assays performed before 2004; batch no. S02909S4190; Bio West Nuaillé, France, for assays after 2004) at a 1.5% hematocrit. ^{3}H-hypoxanthine (1 µCi/well; Amersham International, Little Chalfont, United Kingdom) was added to the blood-serum-medium mixture to measure parasite growth. The suspension (200 µL) was distributed into each well. Parasitemia was adjusted to 0.6% by adding fresh uninfected erythrocytes if the initial parasitemia was ≥ 1%. The culture plates were incubated at 37°C in either a 5% CO_2 incubator (before 2004) or candle jar (after 2004) for 42 hours. The plates were frozen to terminate the *in vitro* assay. Incorporation of ^{3}H-hypoxanthine was measured with a liquid scintillation counter (Wallac 1409; Pharmacia, Uppsala, Sweden). The IC_{50}, defined as the drug concentration at which 50% of the incorporation of ^{3}H-hypoxanthine is inhibited compared with that of drug-free control wells, was calculated by a non-linear regression analysis using Prism™ software (GraphPad Software, Inc., San Diego, CA).

Polymerase chain reaction and DNA sequencing. After performing *in vitro* assays, remaining erythrocyte pellet was frozen at –20°C in aliquots of 1–2 mL and stored. Blood samples were thawed to extract parasite (and human) DNA. Infected erythrocytes were suspended in 15 mL of ice-cold lysis buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5 [NET buffer], 0.015% saponin). The lysate was centrifuged at 2,000 × *g* for 10 minutes, and the pellet was transferred to a 1.5-mL microfuge tube and suspended in 500 µL of NET buffer. The mixture was incubated with 1% N-lauroylsarcosine and RNAse A (100 µg/mL) at 37°C for 1 hour, followed by an incubation with proteinase K (200 µg/mL) at 50°C for 1 hour. DNA was extracted three times in equilibrated phenol, pH 8, phenol-chloroform-isoamyl alcohol (v/v/v 25:24:1), and chloroform-isoamyl alcohol (v/v 24:1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in 50–100 µL of TE buffer (10 mM Tris, 1 mM EDTA).

The complete DNA sequence of the *Pfatp6* gene is 4,049-basepairs (bp), and it is located in chromosome 1. The gene contains three exons and three introns. A 1,793-bp fragment spanning the coding region of exon I of the *Pfatp6* gene was amplified by polymerase chain reaction (PCR). Specific synthetic oligonucleotides were designed from the DNA sequence of the K1 *P. falciparum* strain (GenBank accession no. AB121051). The reaction mixture for the primary PCR consisted of 3 µL of DNA, 15 picomol of the forward primer Pfatp6-1F, 5′-TTTATTTTCATCTACCGCTATTGTATGTGG-3′ and reverse primer Pfatp6-2R, 5′-GCATTTATACATCCTTCT CGTTAATCTAAT-3′, 200 mM of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP, and dCTP), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris buffer, pH 8.4, and 1 unit of *Taq* DNA polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 µL. Amplification was performed using a PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: denaturation at 94°C for 2 minutes for the first cycle and 1 minute in subsequent cycles, annealing at 50°C for 2 minutes for the first cycle and 1 minute in subsequent cycles, and extension at 72°C for 3 minutes for all cycles, for a total of 35 cycles.

The secondary nested PCR was performed with a similar PCR mixture as for the primary amplification using 1 µL of the primary amplification product and 30 picomol of the first internal primer pair Pfatp6-5F (5′-GACTGAAATAGGTCATATTCAGCAT GC-3′) and Pfatp6-6R (5′-CATTTTTATTTTCTTACTATCAC TAG-3′) or the second primer pair Pfatp6-10F (5′-CACCTGTA CAATCATCAAATAAGAAGG-3′) and Pfatp6-12R (5′-CTT CTAATTTATAATAATCATCTGTATTC-3′) in a final volume of 100 µL. The primer pair Pfatp6-5F/Pfatp6-6R yields a 652-bp fragment spanning codons 263 and 431, which were reported to be associated with artemisinin resistance. The primer pair Pfatp6-10F/ Pfatp6-12R amplifies a 645-bp fragment spanning codons 623 and 769. The following thermal cycling program was used for secondary amplification: denaturation at 94°C for 2 minutes for the first cycle and 1 minute for subsequent cycles, annealing at 50°C for 1 minute for all cycles, and extension at 72°C for 1 minute for all cycles, for a total of 35 cycles. The amplified nested PCR products were visualized by agarose gel electrophoresis and ultraviolet transillumination. Each secondary amplification product was sequenced from the 5′- and 3′-ends by using the automated DNA sequencer (ABI Prism; Perkin Elmer Corp., Les Ulis, France).

Data analysis. The *in vitro* sensitivity to artemisinin derivatives was expressed as the geometric mean IC_{50}. The threshold value for artemisinin resistance *in vitro* is not established. The mean IC_{50}s during three study periods (2001–2002, 2003, and 2006) were compared by the one-way analysis of variance. The geometric mean IC_{50}s for dihydroartemisinin were compared between groups of isolates with different *Pfatp6* haplotypes using the Student *t*-test. The significance level of all statistical tests was set at 0.05.

The deduced amino acid sequences of the isolates from Cameroon were compared with those of *P. falciparum* reference clones 3D7 (GenBank accession no. PFA0310c) and K1 (GeneBank accession no. AB121051). Sequence alignment was performed using MacDNASIS Pro software (Hitachi Software

Engineering Co., Yokohama, Japan). The following amino acid substitutions were analyzed for each isolate: L263E, E431K, A623E, and S769N. The presence of novel amino acid residues was also noted. The amino acid substitution observed in some Thai isolates (I89T) was not studied because it was shown to be unrelated to drug sensitivity.[12]

RESULTS

The *in vitro* responses to dihydroartemisinin were available for 234 isolates from 2001 through 2006 (n = 90 in 2001–2002, n = 93 in 2003, and n = 51 in 2006). *In vitro* data for 2001–2003 have been reported in previous work.[22] The geometric mean IC_{50}s (95% confidence interval [CI], [range]) were 1.29 nM (1.09–1.53 nM [0.220–5.88 nM]) in 2001–2002, 0.585 nM (0.475–0.721 nM [0.074–8.21 nM]) in 2003, and 3.30 nM (2.66–4.07 nM [0.636–18.4 nM]) in 2006. The significantly higher dihydroartemisinin IC_{50}s in 2006 ($P < 0.05$) were caused by the change in the batch of pooled human serum and, to a lesser extent, incubation in a candle jar, rather than in a 5% CO_2 incubator that had been used before 2004 in our laboratory. In 2006, *in vitro* response to artemisinin was also determined in parallel with that of dihydroartemisinin. The mean geometric mean IC_{50} (95% CI [range]) for artemisinin was 5.64 nM (4.61–8.51 nM [1.71–38.1 nM]) in 2006.

The *Pfatp6* sequences of 97 isolates collected in 2002, 2003, and 2006 were determined (Tables 1 and 2). Based on codons (263, 431, 623, and 769) that have been associated with artemisinin resistance in previous studies,[17,18] two major haplotypes have been observed in this study: the wild-type LEAS (n = 60) and a single mutant LKAS (n = 35). An additional amino acid residue (E432K) that has not been previously reported was observed in two isolates. The haplotypes (263, 431, 432, 623, and 769) of these isolates were the single mutant LEKAS and the double mutant LKKAS. The West African-type double mutant (E431K + A623E) or the South American-type single mutation (S769N) was not found among isolates from Cameroon. With the exception of these five codons, sequence analysis showed a highly conserved nucleotide sequence, identical to 3D7 sequence, among all isolates within the regions spanning codons 259–454 and 588–790.

The comparison of geometric mean dihydroartemisinin IC_{50}s of isolates carrying LEAS haplotype (1.55 nM) and those with LKAS haplotype (1.38 nM) showed no significant difference ($P > 0.05$), suggesting that the single amino acid substitution at this position is not associated with artemisinin resistance. Further comparison of dihydroartemisinin IC_{50}s in the subsets of isolates by year (2002–2003 and 2006) showed no significant difference ($P > 0.05$) between the two major haplotypes found in this study. The presence of the novel mutation E432K with or without the E431K change resulted in IC_{50}s (0.498 nM and 3.67 nM) within the range of IC_{50}s of isolates with wild-type *Pfatp6*.

DISCUSSION

The present study suggests a high *in vitro* activity of artemisinin derivatives against clinical isolates of *P. falciparum* from Cameroon over the past 13 years (1994–2006).[22–24] Dihydroartemisinin and artemether IC_{50}s have consistently been < 20 nM at our study site. Other *in vitro* studies based on ^{3}H-hypoxanthine radioisotope assay have shown the high

TABLE 1

In vitro dihydroartemisinin sensitivity of *Plasmodium falciparum* isolates with wild type *Pfatp6* codons, Cameroon*

		PfATP6			
Isolate/year	IC_{50}† (nM)	263	431	623	769
02/2002	2.90	L	E	A	S
04/2002	4.44	L	E	A	S
11/2002	1.52	L	E	A	S
13/2002	2.18	L	E	A	S
27/2002	0.688	L	E	A	S
10/2003	0.098	L	E	A	S
12/2003	5.15	L	E	A	S
14/2003	0.157	L	E	A	S
15/2003	0.619	L	E	A	S
16/2003	0.873	L	E	A	S
17/2003	10.4	L	E	A	S
18/2003	2.408	L	E	A	S
19/2003	0.880	L	E	A	S
22/2003	0.129	L	E	A	S
60/2003	0.150	L	E	A	S
67/2003	0.787	L	E	A	S
73/2003	1.50	L	E	A	S
78/2003	0.294	L	E	A	S
82/2003	0.566	L	E	A	S
85/2003	0.336	L	E	A	S
91/2003	1.90	L	E	A	S
93/2003	0.446	L	E	A	S
103/2003	0.190	L	E	A	S
128/2003	0.338	L	E	A	S
130/2003	1.335	L	E	A	S
131/2003	3.00	L	E	A	S
133/2003	0.587	L	E	A	S
135/2003	0.309	L	E	A	S
136/2003	0.775	L	E	A	S
138/2003	0.278	L	E	A	S
141/2003	0.545	L	E	A	S
145/2003	0.492	L	E	A	S
01/2006	3.16	L	E	A	S
04/2006	2.38	L	E	A	S
07/2006	6.89	L	E	A	S
15/2006	2.81	L	E	A	S
16/2006	3.67	L	E	A	S
20/2006	2.44	L	E	A	S
21/2006	3.68	L	E	A	S
23/2006	5.13	L	E	A	S
25/2006	2.19	L	E	A	S
26/2006	2.82	L	E	A	S
27/2006	5.85	L	E	A	S
33/2006	5.00	L	E	A	S
34/2006	1.85	L	E	A	S
53/2006	1.39	L	E	A	S
56/2006	2.36	L	E	A	S
57/2006	5.14	L	E	A	S
59/2006	4.38	L	E	A	S
64/2006	10.1	L	E	A	S
66/2006	7.83	L	E	A	S
69/2006	4.23	L	E	A	S
70/2006	0.857	L	E	A	S
72/2006	7.98	L	E	A	S
84/2006	4.70	L	E	A	S
87/2006	2.19	L	E	A	S
91/2006	3.17	L	E	A	S
98/2006	5.79	L	E	A	S
105/2006	8.82	L	E	A	S
106/2006	6.01	L	E	A	S
45/2003	3.67	L	E	A	S

* *Pfatp6* = *P. falciparum* ATPase 6. IC_{50} = 50% inhibitory concentration. The amino acid substitutions L263E, E431K, A623E, and S769N have been reported in previous studies.[17,18] Two isolates (45/2003 [wild-type *pfatp6*] and 132/2003 [mutant *pfatp6*]) have a novel amino acid residue E432K.

† Geometric mean IC_{50} (95% confidence interval) for isolates with wild-type *pfatp6* (LEAS haplotype, excluding isolate 45/2003) was 1.55 nM (1.12–2.14 nM).

Table 2

In vitro dihydroartemisinin sensitivity of *Plasmodium falciparum* isolates from Cameroon with mutant *Pfatp6* codons*

		PfATP6			
Isolate/year	IC_{50}† (nM)	263	431	623	769
132/2003	0.498	L	**K**	A	S
03/2002	4.19	L	**K**	A	S
10/2002	4.76	L	**K**	A	S
22/2002	2.26	L	**K**	A	S
11/2003	0.663	L	**K**	A	S
24/2003	0.692	L	**K**	A	S
25/2003	0.255	L	**K**	A	S
75/2003	0.137	L	**K**	A	S
79/2003	0.777	L	**K**	A	S
83/2003	1.26	L	**K**	A	S
87/2003	1.54	L	**K**	A	S
90/2003	0.444	L	**K**	A	S
94/2003	3.76	L	**K**	A	S
97/2003	0.548	L	**K**	A	S
99/2003	0.683	L	**K**	A	S
113/2003	0.125	L	**K**	A	S
114/2003	0.404	L	**K**	A	S
116/2003	0.516	L	**K**	A	S
123/2003	0.514	L	**K**	A	S
126/2003	0.258	L	**K**	A	S
134/2003	0.328	L	**K**	A	S
137/2003	0.371	L	**K**	A	S
02/2006	7.27	L	**K**	A	S
08/2006	4.84	L	**K**	A	S
11/2006	5.74	L	**K**	A	S
14/2006	9.01	L	**K**	A	S
30/2006	9.43	L	**K**	A	S
68/2006	18.4	L	**K**	A	S
71/2006	1.45	L	**K**	A	S
75/2006	2.79	L	**K**	A	S
77/2006	1.45	L	**K**	A	S
79/2006	2.66	L	**K**	A	S
81/2006	1.21	L	**K**	A	S
85/2006	0.636	L	**K**	A	S
90/2006	4.36	L	**K**	A	S
108/2006	1.55	L	**K**	A	S

* *Pfatp6* = *P. falciparum* ATPase 6. IC_{50} = 50% inhibitory concentration. The amino acid substitutions L263E, E431K, A623E, and S769N have been reported in previous studies.[17,18] The mutant codon is in **boldface**. Two isolates (45/2003 [wild-type *pfatp6*] and 132/2003 [mutant *pfatp6*]) have a novel amino acid residue E432K.

† Geometric mean IC_{50} (95% confidence interval) for isolates with mutant *pfatp6* (LKAS haplotype, excluding isolate 132/2003) was 1.38 nM (0.889–2.14 nM).

activity of artemisinin derivatives in Africa.[25–27] Few isolates displayed increased IC_{50}s for artemisinin derivatives > 30 nM, even in Asian countries where these drugs have been used more extensively than in Africa.[18,27–29]

Several recent studies have analyzed *Pfatp6* sequences in clinical isolates. In studies conducted on isolates from Tanzania, all codons at positions 263 (n = 355 isolates), 623 (n = 288) and 769 (n from pooled results = 552) were wild type.[30,31] Among *P. falciparum* isolates from Asia, none had mutations in codons 769 (n = 95 isolates from China and 14 isolates from Cambodia) and 263 (n = 14 isolates from Cambodia).[31,32] In a study based on imported malaria from various African countries,[33] no amino acid substitution was also found at position 263 among 154 isolates, whereas only one fully sensitive isolate (dihydroartemisinin IC_{50} = 0.83 nM) carried the South American-type S769N substitution. Our results are in agreement with these recent studies on the analysis of *Pfatp6* codons 263 and 769 in *P. falciparum* isolates from Africa and Asia.

The West African-type E431K and A623E double substitutions were not analyzed in all previous studies. In the present study, E431K single substitution was found in 37% of isolates from Cameroon. Dahlström and others[31] reported a similar finding, with the presence of E431K (mixed alleles E431K + A623E in 2 isolates) in 31% of samples from Zanzibar and 39% from mainland Tanzania but these investigators did not provide *in vitro* response data to correlate their results. Our data suggest that E431K substitution, without A623E change, is insufficient to decrease *in vitro* sensitivity to dihydroartemisinin, as first observed by Jambou and others.[18] Other *Pfatp6* mutations (both synonymous and non-synonymous) have been reported. This gene seems to be more polymorphic in East Africa (Tanzania), with at least 25 amino acid substitutions, mostly in a very limited number of isolates, and possibly in West Africa (Senegal; exact number and positions of mutations unreported), than in central Africa, but the relevance of these mutations to artemisinin resistance is unknown.[18,31]

Most importantly, two molecular indicators of artemisinin resistance previously found in *P. falciparum* isolates, the South American-type *Pfatp6* S769N and the West African-type *Pfatp6* double mutation E431K + A623E,[18] were absent in isolates from Cameroon collected during 2002–2006. However, the presence of a single E431K mutation, found in approximately one-third of isolates from Cameroon and Tanzania, may be a warning signal that warrants a regular monitoring of these molecular markers and/or *in vitro* activity of artemisinin derivatives.

Interpretations of some recent experimental data imply that artemisinins may interact with target(s) other than Pfatp6/SERCA. In an experimental model using *Toxoplasma gondii*, artemisinin resistance was not associated with SERCA sequence changes or expression level but was associated with alterations in cytosolic calcium.[34,35] Moreover, a study has shown the same *in vitro* activities of enantiomeric pairs of trioxanes (synthetic artemisinin analogs) against *P. falciparum*, leading the investigators to conclude that the iron-dependent endoperoxide activation occurs without stereospecific interaction between the drug and its still unidentified target and that artemisinin does not interact with a specific protein target, including SERCA.[36] More laboratory evidence, including studies on PfATP6 structure and interaction with artemisinins,[37] is needed to confirm the pivotal role that *Pfatp6* may play in artemisinin resistance.

The available *in vitro* and molecular data on artemisinin activity and *Pfatp6* sequences are indirectly supported by the generally high efficacy of ACTs in Africa. Most clinical studies based on a 28-day follow-up and PCR adjustment and distinction between recrudescence and reinfection have reported only a few cases of treatment failures.[7,38] The causes of treatment failures are often ascribed to pharmacokinetic and/or pharmacodynamic variations. In this context, further studies are needed to correlate *Pfatp6* sequence variations, *in vitro* artemisinin response, therapeutic efficacy of ACT (or if allowed, artemisinin monotherapy), and plasmatic drug concentrations. Moreover, as long as there is no documented evidence from the field that *Pfatp6* mutations can fully explain artemisinin resistance, *in vitro* and *in vivo*, research on other candidate drug resistance genes should be pursued.

Received September 26, 2008. Accepted for publication April 12, 2009.

Acknowledgments: We are grateful to Sisters Marie-Solange Oko and Pauline Ngono Mbia and their nursing and laboratory staff at the Catholic missionary Nlongkak dispensary in Yaounde for invaluable help in recruiting patients.

Financial support: This study was supported by the European Union (Redox Antimalarial Drug Discovery [READ-UP] project, con-

tract no. 018602) and Technical Service Agreement, Global Malaria Programme, World Health Organization (Geneva, Switzerland).

Disclaimer: Pascal Ringwald is a staff member of the World Health Organization. This author alone is responsible for the views expressed in this publication, and they do not necessarily represent the decisions, policy, or views of the World Health Organization.

Authors' addresses: Rachida Tahar and Leonardo Basco, Unité de Recherche Maladies Infectieuses et Tropicales Emergentes, Institut de Recherche pour le Développement and Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la Lutte Contre les Endémies en Afrique Centrale, BP 288, Yaoundé, Cameroon, E-mails: rachida.tahar@ird.fr and lkbasco@yahoo.fr. Pascal Ringwald, Antimalarial Drug Resistance, Global Malaria Programme, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland, E-mail: ringwaldp@who.int.

REFERENCES

1. World Health Organization, 2006. *Guidelines for the Treatment of Malaria*. Geneva: World Health Organization, WHO/HTM/MAL/2006.1108.
2. World Health Organization, 2001. *Antimalarial Drug Combination Therapy*. Geneva: World Health Organization, WHO/CDS/RBM/2001.35.
3. White NJ, 1998. Preventing antimalarial drug resistance through combinations. *Drug Resist Updat 1:* 3–9.
4. Brossi A, Venugopalan B, Gerpe LD, Yeh HJC, Flippen-Anderson JL, Buchs P, Luo XD, Milhous W, Peters W, 1988. Arteether, a new antimalarial drug: synthesis and antimalarial properties. *J Med Chem 31:* 645–650.
5. Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O, 2006. Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. *Trends Mol Med 12:* 200–205.
6. Eckstein-Ludwig U, Webb RJ, van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S, 2003. Artemisinins target the SERCA of *Plasmodium falciparum. Nature 424:* 957–961.
7. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, 2008. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med 359:* 2619–2620.
8. Looareesuwan S, Wilairatana P, Vanijanonta S, Pitisuttithum P, Ratanapong Y, Andrial M, 1997. Monotherapy with sodium artesunate for uncomplicated falciparum malaria in Thailand: a comparison of 5- and 7-day regimens. *Acta Trop 67:* 197–205.
9. Afonso A, Hunt P, Cheesman S, Alves AC, Cunha CV, do Rosario V, Cravo P, 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes *atp6* (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), *tctp, mdr1*, and *cg10. Antimicrob Agents Chemother 50:* 480–489.
10. Duraisingh MT, Roper C, Walliker D, Warhurst DC, 2000. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the *pfmdr1* gene of *Plasmodium falciparum. Mol Microbiol 36:* 955–961.
11. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF, 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum. Nature 403:* 906–909.
12. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S, 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet 364:* 438–447.
13. Basco LK, Eldin de Pécoulas P, Le Bras J, Wilson CM, 1996. *Plasmodium falciparum*: molecular characterization of multidrug-resistant Cambodian isolates. *Exp Parasitol 82:* 97–103.
14. Ritchie GY, Mungthin M, Green JE, Bray PG, Hawley SR, Ward SA, 1996. *In vitro* selection of halofantrine resistance in *Plasmodium falciparum* is not associated with increased expression of Pgh1. *Mol Biochem Parasitol 83:* 35–46.
15. Basco LK, Ringwald P, 2002. Molecular epidemiology of malaria in Cameroon. X. Evaluation of *pfmdr1* mutations as genetic markers for resistance to amino alcohols and artemisinin derivatives. *Am J Trop Med Hyg 66:* 667–671.
16. Sisowath C, Ferreira PE, Bustamante LY, Dahlström S, Martensson A, Björkman A, Krishna S, Gil JP, 2007. The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Trop Med Int Health 12:* 736–742.
17. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK, Krishna S, 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Mol Biol 12:* 628–629.
18. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O, 2005. Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet 366:* 1960–1963.
19. Mount DL, Nahlen BL, Patchen LC, Churchill FC, 1989. Adaptations of the Saker-Solomons test: simple, reliable colorimetric field assays for chloroquine and its metabolites in urine. *Bull World Health Organ 67:* 295–300.
20. Basco LK, 2007. *Field Application of in vitro Assays for the Sensitivity of Human Malaria Parasites to Antimalarial Drugs*. Geneva: World Health Organization.
21. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD, 1979. Quantitative assessment of antimalarial activity *in vitro* by a semi-automated microdilution technique. *Antimicrob Agents Chemother 16:* 710–718.
22. Basco LK, Ringwald P, 2007. Molecular epidemiology of malaria in Cameroon. XXIV. Trends of *in vitro* antimalarial drug responses in Yaounde, Cameroon. *Am J Trop Med Hyg 76:* 20–26.
23. Ringwald P, Bickii J, Basco LK, 1996. *In vitro* activity of antimalarials against clinical isolates of *Plasmodium falciparum* in Yaoundé, Cameroon. *Am J Trop Med Hyg 55:* 254–258.
24. Ringwald P, Bickii J, Basco LK, 1999. *In vitro* activity of dihydroartemisinin against clinical isolates of *Plasmodium falciparum* in Yaounde, Cameroon. *Am J Trop Med Hyg 61:* 187–192.
25. Doury JC, Ringwald P, Guelain J, Le Bras J, 1992. Susceptibility of African isolates of *Plasmodium falciparum* to artemisinin (qinghaosu). *Trop Med Parasitol 43:* 197–198.
26. Pradines B, Rogier C, Fusai T, Tall A, Trape JF, Doury JC, 1998. *In vitro* activity of artemether against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs. *Am J Trop Med Hyg 58:* 354–357.
27. Menard D, Matsika-Claquin MD, Djalle D, Yapou F, Manirakiza A, Dolmazon V, Sarda J, Talarmin A, 2005. Association of failures of seven-day courses of artesunate in a non-immune population in Bangui, Central African Republic with decreased sensitivity of *Plasmodium falciparum. Am J Trop Med Hyg 73:* 616–621.
28. Bustos MDG, Gay F, Diquet B, 1994. *In-vitro* tests on Philippine isolates of *Plasmodium falciparum* against four standard antimalarials and four qinghaosu derivatives. *Bull World Health Organ 72:* 729–735.
29. Wongsrichanalai C, Wimonwattrawatee T, Sookto P, Laoboonchai A, Heppner DG, Kyle DE, Wernsdorfer WH, 1999. *In vitro* sensitivity of *Plasmodium falciparum* to artesunate in Thailand. *Bull World Health Organ 77:* 392–398.
30. Mugittu K, Genton B, Mshinda H, Beck HP, 2006. Molecular monitoring of *Plasmodium falciparum* resistance to artemisinin in Tanzania. *Malar J 5:* 126. Available at: http://www.malariajournal.com/content/5/1/126.
31. Dahlström S, Veiga MI, Ferreira P, Mårtensson A, Kaneko A, Andersson B, Björkman A, Gil JP, 2008. Diversity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *Plasmodium falciparum* (PfATP6). *Infect Genet Evol 8:* 340–345.
32. Zhang GQ, Guan Y, Zheng B, Wu S, Tang LH, 2008. No *PfATPase6* S769N mutation found in *Plasmodium falciparum* isolates from China. *Malar J 7:* 122. Available at: http://www.malariajournal.com/content/7/1/122.
33. Cojean S, Hubert V, Le Bras J, Durand R, 2006. Resistance to dihydroartemisinin. *Emerg Infect Dis 12:* 1798–1799.
34. Nagamune K, Beatty WL, Sibley LD, 2007. Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii. Euk Cell 6:* 2147–2156.

35. Nagamune K, Moreno SNJ, Sibley LD, 2007. Artemisinin-resistant mutants of *Toxoplasma gondii* have altered calcium homeostasis. *Antimicrob Agents Chemother 51:* 3816–3823.
36. O'Neill PM, Rawe SL, Borstnik K, Miller A, Ward SA, Bray PG, Davies J, Oh CH, Posner GH, 2005. Enantiomeric 1,2,4-trioxanes display equivalent *in vitro* antimalarial activity versus *Plasmodium falciparum* malaria parasites: implications for the molecular mechanism of action of the artemisinins. *ChemBioChem 6:* 1–8.
37. Jung M, Kim H, Nam KY, No KT, 2005. Three-dimensional structure of *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP6) and docking of artemisinin derivatives to PfATP6. *Bioorg Med Chem Lett 15:* 2994–2997.
38. World Health Organization, 2005. *Susceptibility of Plasmodium falciparum to Antimalarial Drugs. Report on global monitoring 1996–2004*. Geneva, World Health Organization, WHO/HTM/MAL/2005.1103.