

MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XXIV. TRENDS OF IN VITRO ANTIMALARIAL DRUG RESPONSES IN YAOUNDE, CAMEROON

LEONARDO K. BASCO* AND PASCAL RINGWALD

Unité de Recherche "Paludologie Afro-tropicale," Institut de Recherche pour le Développement (IRD) and Laboratoire de Recherche sur le Paludisme, Organisation de Coopération pour la lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé, Cameroon; Global Malaria Programme (GMP), World Health Organization, Geneva, Switzerland

Abstract. *In vitro* response to chloroquine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin was assessed by the radioisotopic microtest in Yaoundé, Cameroon, during 2000–2004 and compared with our previous data obtained during 1996–1999. Based on the cut-off value of 100 nmol/L, 36.3% of isolates were chloroquine-susceptible ($N = 175$; geometric mean IC_{50} , 40.3 nmol/L) and 63.7% were chloroquine-resistant ($N = 307$; geometric mean IC_{50} , 211 nmol/L). There was no significant difference ($P > 0.05$) in the mean IC_{50} s from 1996 to 2004, but a significant linear trend ($P < 0.05$) toward an increased proportion of chloroquine-resistant isolates was observed from 1996 (49%) to 2004 (69%). All chloroquine-susceptible isolates and most chloroquine-resistant isolates were susceptible to monodesethylamodiaquine (i.e., $IC_{50} < 60$ nmol/L). Despite the positive correlation between chloroquine and monodesethylamodiaquine ($r = 0.739$, $P < 0.05$), the IC_{50} s for monodesethylamodiaquine remained stable during 1997–2004, with no increase in the proportion of monodesethylamodiaquine-resistant isolates. Mefloquine, lumefantrine, and dihydroartemisinin were equally active against the chloroquine-susceptible and chloroquine-resistant parasites. The responses to these three drugs were positively correlated, and a significant decrease ($P < 0.05$) in the mean IC_{50} s was observed during the study period compared with our earlier data in 1997–1999, probably because of their inverse relationship with chloroquine response. The *in vitro* results were in general agreement with the *in vivo* response to chloroquine and amodiaquine. *In vitro* drug susceptibility assay is a useful, complementary laboratory tool for determining the trend of response to drugs for which there is still no established molecular marker and may serve as an early warning system for emerging drug resistance.

INTRODUCTION

In the face of an increasing prevalence of chloroquine-resistant *Plasmodium falciparum* infections, alternative antimalarial drugs or drug combinations are required. Until a few years ago, chloroquine had been recommended for the first-line treatment of acute uncomplicated *P. falciparum* infections in central Africa. Sulfadoxine-pyrimethamine, amodiaquine, and quinine had been reserved for second-line or third-line treatment. In Cameroon, our clinical data from various sentinel sites have shown that chloroquine is no longer effective in many parts of the country.¹ Based on these results, the use of chloroquine has been gradually phased out, and its importation has been suspended since 2002. Amodiaquine monotherapy was adopted as the drug of first choice during the transition period between 2002 and 2004. Artesunate-amodiaquine combination was officially adopted as the new drug of choice for all cases of uncomplicated malaria in 2004. However, because of the delay in constituting an adequate stock of artesunate at a reasonable price for local populations, the implementation of this new drug policy is yet to be effective throughout the country.

In the private sector, an increasing number of proprietary drugs, including artemisinin derivatives, amino alcohol derivatives (lumefantrine, mefloquine, halofantrine), and antifolates (chlorproguanil-dapsone, sulfalene-pyrimethamine), either as monotherapy or combinations, have become available in pharmacies with unrestricted access, with or without

prescription. This alarming trend toward an uncontrolled use of new antimalarial drugs, which is expected to exert a continual drug pressure on the parasites, portends for a serious situation in the coming years, with a possible selection of multidrug-resistant parasite strains.

The *in vitro* drug susceptibility assay is one of the laboratory tools to monitor the long-term trends of antimalarial drug susceptibility. As many countries resort to combination therapies to increase treatment efficacy and delay the emergence of drug-resistant parasites, monitoring the efficacy of individual components in drug combinations by *in vitro* drug susceptibility assay and/or molecular markers becomes necessary.² The aim of this study was to set up a long-term monitoring system of the *in vitro* activity of antimalarial drugs that may serve as an early warning system of resistance to established and new drugs. The *in vitro* drug activity was determined against fresh clinical isolates of *P. falciparum* collected at the same sentinel site in Yaoundé, and the response to individual drugs was followed over several years and compared with that of chloroquine.

MATERIALS AND METHODS

Patients. Fresh clinical isolates of *P. falciparum* were collected by venipuncture (5–10 mL of blood) into ethylenediaminetetraacetate (EDTA)-coated tubes from symptomatic patients ≥ 12 years old consulting spontaneously at the Nlongkak Catholic missionary dispensary in Yaoundé, Cameroon, from 1996 to 2004. The inclusion criteria were as follows: parasitemia $\geq 0.1\%$, absence of other *Plasmodium* species, and denial of recent self-medication with an antimalarial drug confirmed by the Saker-Solomons urine test.³ Young children < 12 years of age, pregnant women, and patients presenting signs and symptoms of severe and complicated malaria were excluded. The enrolled patients were treated

* Address correspondence to Leonardo Basco, Unité de Recherche Paludologie Afro-tropicale, Institut de Recherche pour le Développement (IRD) and Laboratoire de Recherche sur le Paludisme, Organisation de Coopération pour la lutte contre les Endémies en Afrique Centrale (OCEAC), BP 288, Yaoundé, Cameroon. E-mail: lkbasco@yahoo.fr

with chloroquine, amodiaquine, sulfadoxine-pyrimethamine, amodiaquine-sulfadoxine-pyrimethamine combination, or quinine. The study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

Drugs. Chloroquine sulfate and chloroquine diphosphate were obtained from Sanofi-Aventis (former Rhone-Poulenc-Rorer, Antony, France) and Sigma Chemical Co. (St Louis, MO), respectively. Monodesethylamodiaquine dihydrochloride and monodesethylamodiaquine diphosphate dihydrate were kindly provided by Parke-Davis (Dakar, Senegal) and Professor S. Ward (Liverpool School of Tropical Medicine, Liverpool, UK) through the courtesy of the World Health Organization (Geneva, Switzerland), respectively. Several batches of mefloquine hydrochloride were obtained from Roche (Basel, Switzerland) and Sapec (Lugano, Switzerland) through the courtesy of the World Health Organization. Lumefantrine was kindly provided by Novartis Pharma (Basel, Switzerland). Several batches of dihydroartemisinin were obtained from Sapec and Shin Poong Pharmaceutical Co. (Seoul, South Korea) through the courtesy of the World Health Organization.

Stock and working solutions of chloroquine and monodesethylamodiaquine salts were prepared in sterile distilled water. Solutions of mefloquine and dihydroartemisinin were prepared in pure methanol. The stock solution of lumefantrine was prepared in a mixture (vol/vol/vol 1:1:1) of linoleic acid-ethanol-Tween 80, and further dilutions were prepared in sterile water, according to the protocol developed by W. Wernsdorfer. Two-fold dilutions were pre-coated in triplicate in 96-well culture plates at the final concentrations of 25–1,600 nmol/L for chloroquine, 7.5–480 nmol/L for monodesethylamodiaquine, 2.5–160 nmol/L for mefloquine, 1.25–80 nmol/L for lumefantrine, and 0.25–16 nmol/L for dihydroartemisinin. For some chloroquine assays, 11 2-fold concentrations ranging from 3.12 to 3,200 nmol/L (final concentrations) were distributed in duplicate in 96-well culture plates. All drug solutions were dried in a laminar flow hood. Our preliminary experiments showed that the *in vitro* activity of lumefantrine dissolved in either linoleic acid-ethanol-Tween 80 mixture and further dilutions in water or in pure methanol and further dilutions in pure methanol is similar.

***In vitro* assay.** The *in vitro* response was determined by the isotopic microtest described by Desjardins and others.⁴ Venous blood samples were transported without ice to our laboratory within 1–2 hours after collection. Infected erythrocytes were washed three times with RPMI 1640 [buffered with 25 mmol/L *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) and 25 mmol/L NaHCO₃] by centrifugation and suspended in the complete RPMI 1640 medium with 10% non-immune type AB⁺ pooled human serum at a 1.5% hematocrit. The suspension (200 μ L) was distributed into each well. Parasitemia was adjusted to 0.6% by adding fresh uninfected erythrocytes if the initial parasitemia was \geq 1%. The culture plates were incubated at 37°C in a 5% CO₂ incubator for 42 hours. Parasite growth was assessed by adding ³H-hypoxanthine (1 μ Ci per well; Amersham International, Buckinghamshire, UK) to the culture medium. The plates were frozen to terminate the *in vitro* assay. The incorporation of ³H-hypoxanthine was quantitated with a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden). The 50% inhibitory concentration (IC₅₀), defined as the drug

concentration at which 50% of the incorporation of ³H-hypoxanthine is inhibited, compared with that of drug-free control wells, was calculated by a nonlinear regression analysis using Prism software (GraphPad Software, San Diego, CA).

Data analysis. Data were expressed as the geometric mean IC₅₀. Based on our previous studies,^{5,6} the arbitrary cut-offs for *in vitro* resistance to chloroquine and monodesethylamodiaquine were fixed at IC₅₀ \geq 100 and \geq 60 nmol/L, respectively. Threshold values for the other drugs are not established. The proportions of isolates displaying chloroquine or monodesethylamodiaquine resistance at different time periods were compared by the χ^2 test for trend. Data were tested for normal distribution using the method of Kolmogorov and Smirnov. The method of Bartlett was used to compare the SDs. If the normality test suggested a Gaussian distribution and a Bartlett test suggested that the differences among the SDs were not statistically significant ($P > 0.05$), the geometric mean IC₅₀s were compared by one-way analysis of variance (ANOVA). If these two conditions were not met, the means were compared using the nonparametric Kruskal-Wallis test. Tukey-Kramer post-test of multiple comparisons was performed if ANOVA indicated a significant difference in the means. The coefficients of correlation of the logarithmic IC₅₀ values of different drugs were calculated by a linear regression analysis. The significance level was fixed at $P < 0.05$.

Data from 1994 and 1995 were not included in this analysis because a slightly different assay method (semi-microtest, instead of microtest) was used in those studies. Most of the data obtained between 1996 and 1999 have been published in our previous works.^{5,7–13}

RESULTS

A total of 684 venous blood samples were collected between 2000 and 2004. Some of the samples ($N = 53$) were not used for drug assays because of low parasitemia $< 0.1\%$, diagnostic error of *Plasmodium* species, and self-medication within 24 hours preceding consultation with drugs that are not detectable by the Saker-Solomons urine assay (mostly sulfadoxine-pyrimethamine, but also artemisinin derivatives). Other samples were tested only for *in vitro* response of pyrimethamine (part of the data published elsewhere¹⁴). Drug assays performed with RPMI 1640 medium supplemented with fetal calf serum were excluded from analysis because IC₅₀s differ considerably from those determined in RPMI-human serum mixture. A large majority of isolates ($> 90\%$) that were used for drug assays with RPMI-human serum mixture developed into schizonts. The numbers of interpretable assays for each drug are presented in Table 1. Identifiable causes of failure of schizont maturation included a recent history of self-medication with antimalarial drugs (at concentrations below the detection level of Saker-Solomons test) and antibiotics (e.g., ciprofloxacin), batches of human serum that did not support parasite growth, bacterial contamination of culture medium or blood samples, and technical errors and problems (e.g., unregulated CO₂ content in the incubator and prolonged power cut).

Chloroquine. During 2000–2004, 482 assays for chloroquine were successfully performed. Based on the threshold value of

TABLE 1

Trends of *in vitro* response of Cameroonian *P. falciparum* isolates between 1996 and 2004

Drug	N	No. of resistant isolates (%) [*]	Geometric mean IC ₅₀ (nmol/L)	95% confidence interval	Range
Chloroquine [†]					
1996	98	48 (49.0)	79.3	62.9–99.8	15.3–619
1997	81	44 (54.3)	97.4	78.0–87.0	12.8–508
1998	83	45 (54.2)	85.9	68.7–108	13.2–416
1999	76	36 (47.4)	84.6	67.1–107	19.8–605
2000	104	65 (62.5)	107	88.3–130	12.6–561
2001	118	70 (59.3)	105	87.9–124	18.5–783
2002	98	64 (65.3)	119	99.5–144	14.0–589
2003	113	74 (65.5)	121	103–142	12.5–566
2004	49	34 (69.4)	145	117–179	30.1–470
Monodesethylamodiaquine [‡]					
1997	81	3 (3.7)	26.4	23.3–29.9	3.17–117
1998	34	1 (2.9)	21.4	17.8–25.5	6.90–63.1
1999	29	1 (3.4)	26.3	19.1–36.2	5.21–115
2001	68	0	24.9	22.1–28.1	6.70–59.7
2002	90	5 (5.6)	29.1	26.1–32.4	9.27–93.3
2003	81	6 (7.4)	27.8	24.5–31.4	3.75–88.9
2004	19	1 (5.3)	33.6	27.1–41.7	8.09–63.1
Mefloquine [§]					
1997	32	–	11.7	9.50–14.6	3.18–32.6
1998	52	–	8.57	6.97–10.5	1.70–36.0
2003	88	–	3.91	3.28–4.66	0.67–24.8
Lumefantrine [¶]					
1997	61	–	11.9	10.4–13.6	3.30–25.6
2003	95	–	9.57	8.63–10.6	1.67–25.2
Dihydroartemisinin					
1997–1998	65	–	1.11	0.957–1.28	0.250–4.56
2001–2002	90	–	1.29	1.09–1.53	0.220–5.88
2003	93	–	0.585	0.475–0.721	0.074–8.21

^{*} The arbitrary thresholds of *in vitro* resistance are ≥ 100 nmol/L for chloroquine and ≥ 60 nmol/L for monodesethylamodiaquine. The thresholds for mefloquine, lumefantrine, and dihydroartemisinin are undetermined.

[†] Data from 1996 to 1999 are from Ringwald and others.¹¹

[‡] Parts of data from 1997 to 1999 have been published elsewhere.^{5,7,8,12,13}

[§] Data from 1997 to 1998 were published elsewhere.^{7,8}

[¶] Data obtained in 1997 were published in Basco and others.⁷

^{||} Data from 1997 to 1998 and 2001 to 2002 were published elsewhere.^{9,13}

100 nmol/L, 175 (36.3%) isolates were chloroquine-susceptible, with a geometric mean IC₅₀ of 40.3 nmol/L (range, 12.5–99.2 nmol/L; 95% confidence intervals, 37.4–43.4 nmol/L), and 307 (63.7%) isolates were chloroquine-resistant, with a geometric mean IC₅₀ of 211 nmol/L (range, 101–784 nmol/L; 95% confidence intervals, 201–221 nmol/L). The annual geometric mean IC₅₀s during the study period did not differ significantly ($P > 0.05$). Compared with the previous results obtained during 1996–1999, there was no significant difference in the mean IC₅₀s ($P > 0.05$). There was no significant ($P > 0.05$) linear trend in the proportions of chloroquine-resistant isolates during the study period between 2000 and 2004. However, when our earlier data are included, a significant linear trend ($P < 0.05$) toward an increased proportion of chloroquine-resistant isolates from 1996 (49%) to 2004 (69%) was observed.

Monodesethylamodiaquine. The *in vitro* response to monodesethylamodiaquine (also mefloquine, lumefantrine, and dihydroartemisinin) was assessed in a subset of isolates collected during the study period. Of 258 isolates studied for both chloroquine and monodesethylamodiaquine responses in 2001–2004, 100 (38.8%) were chloroquine-susceptible, with geometric mean IC₅₀s of 41.4 nmol/L (range, 7.05–98.8 nmol/L; 95% confidence interval, 37.1–46.2 nmol/L) and 18.9 nmol/L (range, 3.75–58.5 nmol/L; 95% confidence

interval, 17.1–20.9 nmol/L) for chloroquine and monodesethylamodiaquine, respectively. The other 158 (61.2%) isolates were chloroquine-resistant, with geometric mean IC₅₀s of 188 nmol/L (range, 101–566 nmol/L; 95% confidence interval, 177–200 nmol/L) and 35.5 nmol/L (range, 16.6–93.3 nmol/L; 95% confidence interval, 33.6–37.6 nmol/L) for chloroquine and monodesethylamodiaquine, respectively. Monodesethylamodiaquine was, on the average, 1.9-fold more active (unpaired *t* test, $P < 0.05$) against the chloroquine-susceptible isolates than against the chloroquine-resistant parasites. The response to chloroquine and monodesethylamodiaquine was highly correlated ($r = 0.739$, $N = 80$ in 2003, $P < 0.05$; $r = 0.756$, $N = 400$ in 1998–2003, $P < 0.05$; Figure 1). All chloroquine-susceptible isolates ($N = 100$), as well as most chloroquine-resistant isolates (145 of 158; 92%), displayed monodesethylamodiaquine IC₅₀ < 60 nmol/L. Of 12 isolates with monodesethylamodiaquine IC₅₀ ≥ 60 nmol/L, 11 were highly chloroquine-resistant (range, IC₅₀ 252–566 nmol/L), whereas one isolate showed a borderline resistance (IC₅₀, 109 nmol/L). Monodesethylamodiaquine dihydrochloride and diphosphate dihydrate salts yielded closely similar IC₅₀s (data not shown). The annual mean IC₅₀s between 2001 and 2004 did not differ significantly ($P > 0.05$). Likewise, the IC₅₀s during 2001–2004 and those during 1997–1999 grouped together ($N = 144$) did not differ significantly ($P > 0.05$). There was no linear trend ($P > 0.05$) in the proportions of resistant isolates with monodesethylamodiaquine IC₅₀ ≥ 60 nmol/L.

Mefloquine. The *in vitro* activity of mefloquine was assessed in 88 isolates in 2003. The mean mefloquine IC₅₀ of 28 chloroquine-susceptible isolates was 3.30 nmol/L (range, 0.67–23.0 nmol/L; 95% confidence interval, 2.43–4.46 nmol/L). For 60 chloroquine-resistant isolates, the geometric mean mefloquine IC₅₀ was 4.22 nmol/L (range, 0.720–24.8 nmol/L; 95% confidence interval, 3.40–5.23 nmol/L). The geometric means of these two groups did not differ significantly (unpaired *t* test, $P > 0.05$). Compared with our 1997–1998 data, there was a significant decrease ($P < 0.05$) in the mean IC₅₀.

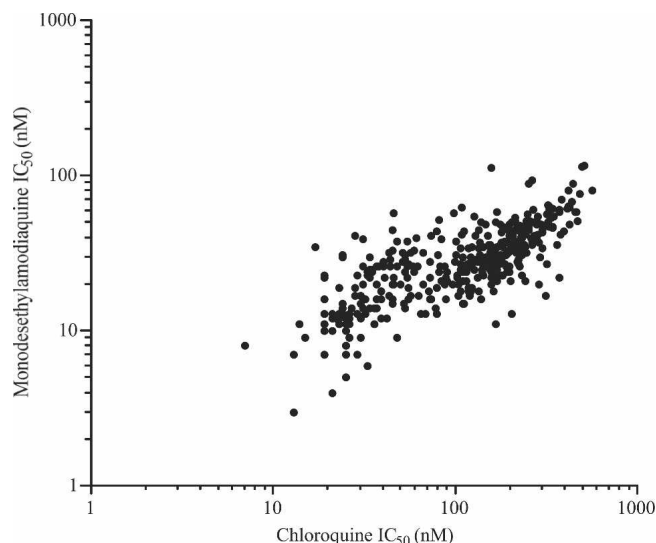


FIGURE 1. Positive correlation of *in vitro* response to chloroquine and monodesethylamodiaquine ($r = 0.756$, $P < 0.05$). Data from 1998 to 2003 were included in this graph.

The responses of mefloquine and chloroquine were not correlated ($r = 0.184$, $N = 88$ in 2003, $P > 0.05$; $r = -0.076$, $N = 105$ in 1997–2003, $P > 0.05$).

Lumefantrine. In 2003, *in vitro* lumefantrine activity was evaluated in 95 isolates. Twenty-nine chloroquine-susceptible isolates had a geometric mean IC_{50} for lumefantrine of 8.27 nmol/L (range, 1.67–22.8 nmol/L; 95% confidence interval, 6.43–10.6 nmol/L), whereas 66 chloroquine-resistant isolates had a geometric mean IC_{50} of 10.2 nmol/L (range, 4.41–25.2 nmol/L; 95% confidence interval, 9.23–11.3 nmol/L). The *in vitro* activity of lumefantrine against the chloroquine-susceptible and the chloroquine-resistant parasites did not differ (unpaired *t* test, $P > 0.05$). Although the geometric means were close, there was a significant difference ($P < 0.05$) between the values obtained in 1997 (11.9 nmol/L) and in 2003 (9.57 nmol/L). Lumefantrine and mefloquine IC_{50} s were correlated ($r = 0.328$, $N = 87$ in 2003, $P < 0.05$; $r = 0.394$, $N = 119$ in 1997 and 2003, $P < 0.05$).

Dihydroartemisinin. Among 93 isolates tested for *in vitro* response to dihydroartemisinin in 2003, 29 were chloroquine-susceptible (geometric mean IC_{50} for dihydroartemisinin, 0.704 nmol/L; range, 0.125–8.21 nmol/L; 95% confidence interval, 0.469–1.06 nmol/L) and 64 were chloroquine-resistant (geometric mean IC_{50} for dihydroartemisinin, 0.538 nmol/L; range, 0.074–4.64 nmol/L; 95% confidence interval, 0.421–0.688 nmol/L). The difference between the mean IC_{50} s of the chloroquine-susceptible and the chloroquine-resistant isolates was not significant (unpaired *t* test, $P > 0.05$). While there was no significant difference ($P > 0.05$) in the means determined in 1997–1998 and 2001–2002, there was a significant decrease ($P < 0.05$) in the mean IC_{50} in 2003. There was a weak, positive correlation between the responses of dihydroartemisinin and mefloquine ($r = 0.233$, $N = 80$ in 2003, $P < 0.05$; $r = 0.384$ in 1997–2003, $N = 105$, $P < 0.05$) and between the responses of dihydroartemisinin and lumefantrine ($r = 0.308$, $N = 86$ in 2003, $P < 0.05$). The responses of dihydroartemisinin and chloroquine were not correlated ($r = 0.026$, $N = 93$ in 2003, $P > 0.05$; $r = 0.006$, $N = 248$ in 1997–2003, $P > 0.05$).

DISCUSSION

In our earlier study,¹¹ the level of *in vitro* chloroquine response was shown to be stable between 1994 and 1999 in Yaoundé. This study confirms the stability of chloroquine resistance from 2000 to 2004, but the proportion of chloroquine-resistant isolates was higher than in our earlier study (51.2% during 1996–1999 versus 63.7% during 2000–2004). The comparison based on the proportions of chloroquine-resistant isolates (i.e. $IC_{50} \geq 100$ nmol/L) may reflect more accurately the trend of resistance because the difference of IC_{50} s between the chloroquine-susceptible and the chloroquine-resistant isolates may be 5- to 10-fold and the 95% confidence intervals of geometric mean IC_{50} for chloroquine are wide unless the chloroquine-susceptible and the chloroquine-resistant isolates are analyzed separately.

The use of chloroquine for treatment and prophylaxis in pregnant women had been intensive throughout the country until 2002, when the Cameroonian government decided to halt importation of the drug because of its sharply declining clinical efficacy. Even during 2003–2004, health care facilities were authorized to prescribe the remaining stock of chloro-

quine without replenishment of supply. Moreover, chloroquine has been widely available through unofficial outlets for self-medication. Although a substantial proportion of chloroquine available in the informal sector is of substandard quality, the massive consumption of this drug in most households have been maintaining drug pressure on malaria parasites despite recent changes in national policy for antimalarial drug use.¹⁵

Our previous study suggested that there is a moderate concordance between the *in vitro* response and clinical outcome in patients treated with chloroquine and that the predictive value of the *in vitro* test for distinguishing between the chloroquine-susceptible and the chloroquine-resistant cases is 86%.¹⁰ Furthermore, the evaluation of the clinical efficacy of chloroquine conducted between 1999 and 2002 has shown that chloroquine treatment is ineffective in most regions of Cameroon, resulting in 48.6% failure rate.¹ There is a general agreement between the observed proportion of chloroquine-resistant isolates *in vitro* and the poor clinical response to chloroquine therapy. Furthermore, the *in vitro* response of Cameroonian isolates to chloroquine is highly concordant with the key codon of the *P. falciparum* chloroquine-resistance transporter (*pfcr*) gene, and the threshold of chloroquine $IC_{50} \geq 100$ nmol/L distinguished between isolates carrying the wild-type *pfcr* alleles and those with mutant *pfcr* alleles in a large majority of parasites.¹⁶ Because it has become inappropriate and unethical to treat falciparum malaria with chloroquine in Cameroon, future surveillance of chloroquine-resistant *P. falciparum* may be performed by either *in vitro* drug susceptibility assay or molecular analysis.

Our results suggest that monodesethylamodiaquine retains its high *in vitro* activity against both chloroquine-susceptible parasites and a large majority of chloroquine-resistant *P. falciparum* isolates. The *in vitro* data obtained during 2001–2004 are similar to those of 1994–1997 and 1998–1999 in Yaoundé.^{5,7,8} Although *in vitro* response cannot be directly extrapolated to predict *in vivo* response, our *in vitro* results are in agreement with the high clinical efficacy of amodiaquine in Cameroon. In a study conducted in Yaoundé in 1997–1999, there was no treatment failure in older children > 5 years old and adults during the 14-day follow-up period.¹² In another study in the coastal area of Cameroon in 2001, late treatment failure was observed in only 3.3% (sample size, 61 children < 5 years old) and 10.2% during the 14- and 28-day follow-up periods, respectively.¹⁷ In Cameroon, where most regions have become highly chloroquine-resistant (mean failure rate on day 14, 48.6%), the overall failure rate after amodiaquine monotherapy was 7.3% during the 14-day follow-up.¹

The high clinical efficacy of amodiaquine monotherapy has been shown in other parts of Africa.^{18,19} Its efficacy to eliminate chloroquine-resistant parasites is of utmost importance for malaria control programs in Africa. Amodiaquine efficacy needs to be closely monitored. Elsewhere in the world where chloroquine, a 4-aminoquinoline, has become almost totally ineffective for cure, amodiaquine (a Mannich-base derivative of 4-aminoquinoline) is also ineffective because of cross-resistance.^{20–22} *In vitro* studies, including this study, also support the existence of cross-resistance between these two drugs.^{23–25} Cross-resistance between chemically similar drugs is a source of concern for the long-term viability of amodiaquine administered as monotherapy in chloroquine-resistant

zones. In Cameroon, the use of amodiaquine monotherapy has been limited at the national level. It is most probably unrelated with the selection of chloroquine-resistant parasites. Rational drug use and combination therapies, including amodiaquine-sulfadoxine-pyrimethamine and amodiaquine-artesunate, are possible strategies to delay the emergence and spread of amodiaquine resistance in Africa.^{17,26,27} If a combination therapy including amodiaquine is to be massively deployed to combat against chloroquine-resistant *P. falciparum* in Africa, as planned by several countries,² we need to be assured of the high efficacy of amodiaquine to maintain mutual protection of drug partners.

Mefloquine and lumefantrine, as well as quinine and halofantrine, belong to the amino alcohol class. Dihydroartemisinin is a sesquiterpene lactone derivative. Since several years ago, these drugs have been available at the pharmacies in Cameroon, either as a combination (mefloquine-artesunate, lumefantrine-artemether, amodiaquine-artesunate) or monotherapy (dihydroartemisinin, artesunate, artemether). The role of mefloquine-artesunate combination is yet to be defined. Artemisinin derivatives as a monotherapy do not seem to have any particular role in malaria control in Africa,²⁸ except for the parenteral forms of artemisinin derivatives that may be useful as an alternative emergency treatment of severe and complicated malaria. In contrast, lumefantrine-artemether combination was added to the World Health Organization list of essential drugs. National malaria control programs may advocate its use in the coming years. Since 2006, lumefantrine-artemether combination is recommended as an alternative to artesunate-amodiaquine combination in Cameroon.

The activity of these drugs was assessed in 2003 (also in 2001–2002 for dihydroartemisinin). They were equally active against the chloroquine-susceptible and the chloroquine-resistant parasites. The positive correlation of response between mefloquine and lumefantrine suggests *in vitro* cross-resistance, as expected for drugs belonging to the same chemical class. *In vitro* cross-resistance between amino alcohols and dihydroartemisinin (or other artemisinin derivatives) was less expected but already observed in several *in vitro* studies.^{29–33} An identical drug transport process within the parasite or parasite-infected erythrocyte may explain, at least in part, cross-resistance between amino alcohols and artemisinin derivatives, but its exact underlying mechanism is unknown.^{34,35}

The 2003 data confirm the results of our earlier studies conducted in 1997–1998.^{7–9} For mefloquine, lumefantrine, and dihydroartemisinin, a statistically significant decrease in the mean IC_{50} s was observed in 2003, as compared with our earlier data from 1997–1998. The most likely explanation is an inverse relationship between the responses of chloroquine and amino alcohols, suggested by several *in vitro* studies,^{30,36–38} which may have brought about a decrease in the mean IC_{50} s of amino alcohols and dihydroartemisinin in the face of an increased proportion of chloroquine-resistant isolates in 2000–2004. To prove this possibility, further monitoring of *in vitro* drug response of isolates is required. Other possible technical causes of decreased IC_{50} s for amino alcohol and artemisinin derivatives may include the use of different batches of active principles (with the exception of lumefantrine, for which only one batch was available), different batches of pooled human serum, and assay plate preparation. The use of several batches of active principles of mefloquine

and dihydroartemisinin is an unlikely source of variations in IC_{50} s. The use of human serum proved to pose a problem in 2003 when the previous batch of pooled serum obtained from the Blood Transfusion Center (Strasbourg, France) and used for all our drug assays performed during 1996–2002 had to be replaced with a new batch of serum. Several batches purchased from commercial sources did not support parasite growth. A satisfactory *in vitro* growth was obtained with pooled sera from several hundreds of donors provided by a commercial supplier. The protocols for plate preparation remained essentially the same in our laboratory since 1996. However, since a few years ago, smaller quantities of pre-dosed plates are prepared weekly to avoid drug degradation during prolonged storage. These potential sources of laboratory artefacts highlight the need for a standardized protocol for *in vitro* drug susceptibility assays and for a suitable serum substitute with no batch-to-batch variation of parasite growth and drug response.

Combination therapy is probably the best strategy at present to prolong the useful lifespan of drugs that are still effective in most of Africa, such as amodiaquine and sulfadoxine-pyrimethamine. A regular surveillance of the *in vitro* drug response and/or molecular markers is warranted as most countries resort to drug combinations for the treatment of uncomplicated falciparum malaria. *In vitro* drug susceptibility assay is a laboratory tool that can determine the parasite's response to individual drugs without interference of host factors. One of the technical limitations of the *in vitro* assay is that the phenotype is largely determined by the predominant parasite population(s) adapting to *in vitro* culture. In Yaoundé, the majority of fresh isolates are composed of multiple parasite populations.^{39,40} If equal proportions of drug-susceptible parasites and drug-resistant parasites are cultivated, the resulting IC_{50} may tend to reflect the response of the resistant parasites. Another limitation is that the *in vitro* assay applied in the field is designed to measure the response to single drugs. *In vitro* studies of synergy or additivity by isobologram require the prior knowledge of IC_{50} s of individual drugs for a given isolate and evaluation of several fixed combinations of the two drugs. Such experimental studies are rarely performed in the field. Furthermore, *in vitro* assays of a single fixed drug combination are usually difficult to interpret since drug concentrations are fixed arbitrarily. Despite these technical limitations, *in vitro* drug susceptibility assay remains an important component of the research on and surveillance of drug-resistant malaria since there are still no well-established molecular markers associated with resistance to amodiaquine, amino alcohols, and artemisinin derivatives.

Received March 23, 2006. Accepted for publication September 4, 2006.

Acknowledgment: The authors thank the staff of Nlongkak Catholic missionary dispensary for recruitment of patients.

Financial support: This study was supported by the French Ministry of Research (Programme PAL+) and the World Health Organization, Geneva, Switzerland (contract C8/181/32).

Authors' addresses: Leonardo K. Basco, Unité de Recherche Paludologie Afro-tropicale, Institut de Recherche pour le Développement (IRD) and Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), BP 288, Yaoundé, Cameroon. Telephone: 237-223-22-32, Fax: 237-223-00-61, E-mail: lkbasco@yahoo.fr. Pascal Ringwald, Global Malaria Programme, World Health Organization,

20 Avenue Appia, 1211 Geneva 27, Switzerland, Telephone: 41-22-7913469, Fax: 41-22-7914824, E-mail: ringwaldp@who.int.

REFERENCES

- Basco LK, Foumane Ngane V, Ndounga M, Same-Ekobo A, Youmba JC, Okalla Abodo RT, Soula G, 2006. Molecular epidemiology of malaria in Cameroon. XXI. Baseline therapeutic efficacy of chloroquine, amodiaquine, and sulfadoxine-pyrimethamine monotherapies in children before national drug policy change. *Am J Trop Med Hyg* 75: 388–395.
- World Health Organization, 2005. *Susceptibility of Plasmodium falciparum to Antimalarial Drugs. Report on Global Monitoring 1996–2004*. Geneva: World Health Organization.
- 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.
- 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.
- Ringwald P, Bickii J, Basco LK, 1998. Amodiaquine as the first-line treatment of malaria in Yaoundé, Cameroon: presumptive evidence from activity *in vitro* and cross-resistance patterns. *Trans R Soc Trop Med Hyg* 92: 212–213.
- Basco LK, Ndounga M, Keundjian A, Ringwald P, 2002. Molecular epidemiology of malaria in Cameroon. IX. Characteristics of recrudescence and persistent *Plasmodium falciparum* infections after chloroquine or amodiaquine treatment in children. *Am J Trop Med Hyg* 66: 117–123.
- Basco LK, Bickii J, Ringwald P, 1998. *In vitro* activity of lumefantrine (benflumetol) against clinical isolates of *Plasmodium falciparum* in Yaoundé, Cameroon. *Antimicrob Agents Chemother* 42: 2347–2351.
- Ringwald P, Eboumbou ECM, Bickii J, Basco LK, 1999. *In vitro* activity of pyronaridine, alone and in combination with other antimalarial drugs, against *Plasmodium falciparum*. *Antimicrob Agents Chemother* 43: 1525–1527.
- Ringwald P, Bickii J, Basco LK, 1999. *In vitro* activity of dihydroartemisinin against clinical isolates of *Plasmodium falciparum* in Yaoundé, Cameroon. *Am J Trop Med Hyg* 61: 187–192.
- Ringwald P, Basco LK, 1999. Comparison of *in vivo* and *in vitro* tests of resistance in patients treated with chloroquine in Yaoundé, Cameroon. *Bull World Health Organ* 77: 34–43.
- Ringwald P, Same Ekobo A, Keundjian A, Kedy Mangamba D, Basco LK, 2000. Chimiorésistance de *P. falciparum* en milieu urbain à Yaoundé, Cameroun. Part I: Surveillance *in vitro* et *in vivo* de la résistance de *Plasmodium falciparum* à la chloroquine entre 1994 et 1999 à Yaoundé, Cameroun. *Trop Med Int Health* 5: 612–619.
- Ringwald P, Keundjian A, Same-Ekobo A, Basco LK, 2000. Chimiorésistance de *P. falciparum* en milieu urbain à Yaoundé, Cameroun. Part 2: Evaluation de l'efficacité de l'amodiaquine et de l'association sulfadoxine-pyriméthamine pour le traitement de l'accès palustre simple à *Plasmodium falciparum* à Yaoundé, Cameroun. *Trop Med Int Health* 5: 620–627.
- Basco LK, Ringwald P, 2003. *In vitro* activities of piperazine and other 4-aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrob Agents Chemother* 47: 1391–1394.
- Basco LK, 2003. Molecular epidemiology of malaria in Cameroon. XVI. Longitudinal surveillance of *in vitro* pyrimethamine resistance. *Am J Trop Med Hyg* 69: 174–178.
- Basco LK, 2004. Molecular epidemiology of malaria in Cameroon. XIX. Quality of antimalarial drugs used for self-medication. *Am J Trop Med Hyg* 70: 245–250.
- Basco LK, 2002. Molecular epidemiology of malaria in Cameroon. XIII. Analysis of *pfcr* mutations and *in vitro* chloroquine resistance. *Am J Trop Med Hyg* 67: 388–391.
- Basco LK, Same-Ekobo A, Foumane Ngane V, Ndounga M, Me-toh T, Ringwald P, Soula G, 2002. Therapeutic efficacy of sulfadoxine-pyrimethamine, amodiaquine, and sulfadoxine-pyrimethamine-amodiaquine combination for the treatment of uncomplicated *Plasmodium falciparum* malaria in young children in Cameroon. *Bull World Health Organ* 80: 538–545.
- Olliaro P, Nevill C, Le Bras J, Ringwald P, Mussano P, Garner P, Brasseur P, 1996. Systematic review of amodiaquine treatment in uncomplicated malaria. *Lancet* 348: 1196–1201.
- Brasseur P, Guiguemde R, Diallo S, Guiyedi V, Kombila A, Ringwald P, Olliaro P, 1999. Amodiaquine remains effective for treating uncomplicated malaria in West and Central Africa. *Trans R Soc Trop Med Hyg* 93: 645–650.
- Hall AP, Segal HE, Pearlman EJ, Phintuyothin P, Kosakal S, 1975. Amodiaquine resistant falciparum malaria in Thailand. *Am J Trop Med Hyg* 24: 575–580.
- Pinichpongse S, Doberstyn EB, Cullen JR, Yisunri L, Thongsombun Y, Thimarsan K, 1982. An evaluation of five regimens for the outpatient therapy of falciparum malaria in Thailand 1980–81. *Bull World Health Organ* 60: 907–912.
- Khalid AA, Fox E, Sarwar M, Strickland GT, 1987. Amodiaquine fails to cure chloroquine-resistant *Plasmodium falciparum* in the Punjab. *Trans R Soc Trop Med Hyg* 81: 157–159.
- Le Bras J, Simon F, Ramanamirija JA, Calmel MB, Hatin I, Deloron P, Porte J, Marchais H, Clause JL, Biau JM, Sarrouy J, Guiguemde TR, Carne B, Charmot G, Coulaud JP, Coulanges P, 1987. Sensibilité de *Plasmodium falciparum* aux quinoléines et stratégies thérapeutiques: comparaison de la situation en Afrique et à Madagascar entre 1983 et 1986. *Bull Soc Pathol Exot* 80: 477–489.
- Childs GE, Boudreau EF, Milhous WK, Wimonwatrattee T, Pooyindee N, Pang L, Davidson DE, 1989. A comparison of the *in vitro* activities of amodiaquine and desethylamodiaquine against isolates of *Plasmodium falciparum*. *Am J Trop Med Hyg* 40: 7–11.
- Basco LK, Le Bras J, 1993. *In vitro* activities of monodesethylamodiaquine and amopyroquine against African isolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* 48: 120–125.
- Adjuik M, Agnamey P, Babiker A, Borrmann S, Brasseur P, Cisse M, Cobelens F, Diallo S, Faucher JF, Garner P, Gikunda S, Kreamsner PG, Krishna S, Lell B, Loolpait M, Matsiegui PB, Missinou MA, Mwanza J, Ntoumi F, Olliaro P, Osimbo P, Rezbach P, Some E, Taylor WR, 2002. Amodiaquine-artesunate versus amodiaquine for uncomplicated *Plasmodium falciparum* malaria in African children: a randomised, multicentre trial. *Lancet* 359: 1365–1372.
- Dorsey G, Njama D, Kanya MR, Cattamanchi A, Kyabayinze D, Staedke SG, Gasasira A, Rosenthal PJ, 2002. Sulfadoxine/pyrimethamine alone or with amodiaquine or artesunate for treatment of uncomplicated malaria: a longitudinal randomised trial. *Lancet* 360: 2031–2038.
- Bohannon J, 2006. Profile: Arata Kochi. Fighting words from WHO's new malaria chief. *Science* 311: 599.
- 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.
- Basco LK, Le Bras J, 1993. *In vitro* activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* 49: 301–307.
- 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.
- 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.
- Tanariya P, Tippawangkoson P, Karbwang J, Na-Bangchang K, Wernsdorfer WH, 2000. *In vitro* sensitivity of *Plasmodium falciparum* and clinical response to lumefantrine (benflumetol) and artemether. *Br J Clin Pharmacol* 49: 437–444.
- 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.
- Duraisingh MT, Roper C, Walliker D, Warhurst DC, 2000. Increased sensitivity to the antimalarials mefloquine and arte-

- misinin is conferred by mutations in the *pfmdr1* gene of *Plasmodium falciparum*. *Mol Microbiol* 36: 955–961.
36. Oduola AMJ, Milhous WK, Salako LA, Walker O, Desjardins RE, 1987. Reduced *in vitro* susceptibility to mefloquine in West African isolates of *Plasmodium falciparum*. *Lancet* 2: 1304–1305.
 37. Bossi A, Venugopalan B, Dominguez-Gerpe L, 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.
 38. Simon F, Le Bras J, Gaudebout C, Girard PM, 1988. Reduced sensitivity of *Plasmodium falciparum* to mefloquine in West Africa. *Lancet* 1: 467–468.
 39. Basco LK, Ringwald P, 2001. Molecular epidemiology of malaria in Yaounde, Cameroon. VIII. Multiple *Plasmodium falciparum* infections in symptomatic patients. *Am J Trop Med Hyg* 65: 798–803.
 40. Basco LK, Tahar R, Escalante A, 2004. Molecular epidemiology of malaria in Cameroon. XVIII. Polymorphisms of the *Plasmodium falciparum* merozoite surface antigen-2 gene in isolates from symptomatic patients. *Am J Trop Med Hyg* 70: 238–244.

Basco Leonardo, Ringwald Pascal (2007)

Molecular epidemiology of malaria in Cameroon: 24. Trends of in vitro antimalarial drug responses in Yaoundé, Cameroon

American Journal of Tropical Medicine and Hygiene, 76 (1), 20-26

ISSN 0002-9637