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 Coordination 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 IC₅₀, 40.3 nmol/L) and 63.7% were chloroquine-resistant (N = 307; geometric mean IC₅₀, 211 nmol/L). There was no significant difference (P > 0.05) in the mean IC₅₀ 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., IC₅₀ < 60 nmol/L). Despite the positive correlation between chloroquine and monodesethylamodiaquine (r = 0.739, P < 0.05), the IC₅₀ 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 IC₅₀ 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 results 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. 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 continuous 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. 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 Nongkak Catholic mission hospital in Yaoundé, Cameroon, from 1996 to 2004. The inclusion criteria were as follows: 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. 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...
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 dihydride 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.² 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 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 a 5% CO₂ incubator for 42 hours. Parasite growth was assessed by adding ³H-hypoxanthine (1 μCi 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,⁶ the arbitrary cut-offs for in vitro resistance to chloroquine and monodesethylamodiaquine were fixed at IC₅₀ ≥ 100 and ≥ 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 χ² 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.⁵⁷–¹³

**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 artesiminin 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
100 nmol/L, 175 (36.3%) isolates were chloroquine-susceptible, with a geometric mean IC$_{50}$ 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 IC$_{50}$ of 211 nmol/L (range, 101–784 nmol/L; 95% confidence intervals, 201–221 nmol/L). The annual geometric mean IC$_{50}$ 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 IC$_{50}$ ($P > 0.05$). 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 a geometric mean IC$_{50}$ 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 IC$_{50}$ 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 IC$_{50} < 60$ nmol/L. Of 12 isolates with monodesethylamodiaquine IC$_{50} \geq 60$ nmol/L, 11 were highly chloroquine-resistant (range, IC$_{50}$ 252–566 nmol/L), whereas one isolate showed a borderline resistance (IC$_{50}$ 109 nmol/L). Monodesethylamodiaquine dihydrochloride and diphosphate hydrate salts yielded closely similar IC$_{50}$ (data not shown). The annual mean IC$_{50}$ between 2001 and 2004 did not differ significantly ($P > 0.05$). Likewise, the IC$_{50}$ 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 IC$_{50} \geq 60$ nmol/L.

**Mefloquine.** The *in vitro* activity of mefloquine was assessed in 88 isolates in 2003. The mean mefloquine IC$_{50}$ 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 IC$_{50}$ 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 IC$_{50}$.
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\(_{50}\) 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\(_{50}\) 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 \((i.e. IC_{50} \geq 100\ \text{nmol/L})\) may reflect more accurately the trend of resistance because the difference of IC\(_{50}\) 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 chloroquine 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 codons of the \(P. falciparum\) chloroquine-resistance transporter \((pfcr)\) gene, and the threshold of chloroquine IC\(_{50} \geq 100\ \text{nmol/L}\) 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.\(^{13}\) 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 chloroquine resistance in Africa.\textsuperscript{17,26,27} If a combination therapy including amodiaquine is to be massively deployed to combat against chloroquine-resistant \textit{P. falciparum} in Africa, as planned by several countries,\textsuperscript{7} 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,\textsuperscript{28} except for the parental 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 \textit{in vitro} cross-resistance, as expected for drugs belonging to the same chemical class. \textit{In vitro} cross-resistance between amino alcohols and dihydroartemisinin (or other artemisinin derivatives) was less expected but already observed in several \textit{in vitro} studies.\textsuperscript{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.\textsuperscript{34,35}

The 2003 data confirm the results of our earlier studies conducted in 1997–1998.\textsuperscript{7–9} For mefloquine, lumefantrine, and dihydroartemisinin, a statistically significant decrease in the mean IC\textsubscript{50} 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 \textit{in vitro} studies,\textsuperscript{30,36–38} which may have brought about a decrease in the mean IC\textsubscript{50} 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 \textit{in vitro} drug response of isolates is required. Other possible technical causes of decreased IC\textsubscript{50} 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\textsubscript{50}. 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 \textit{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 predosed 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 \textit{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 \textit{in vitro} drug response and/or molecular markers is warranted as most countries resort to drug combinations for the treatment of uncomplicated falciparum malaria. \textit{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 \textit{in vitro} assay is that the phenotype is largely determined by the predominant parasite population(s) adapting to \textit{in vitro} culture. In Yaoundé, the majority of fresh isolates are composed of multiple parasite populations.\textsuperscript{39,40} If equal proportions of drug-susceptible parasites and drug-resistant parasites are cultivated, the resulting IC\textsubscript{50} may tend to reflect the response of the resistant parasites. Another limitation is that the \textit{in vitro} assay applied in the field is designed to measure the response to single drugs. \textit{In vitro} studies of synergy or additivity by isobologram require the prior knowledge of IC\textsubscript{50} 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, \textit{in vitro} assays of a single fixed drug combination are usually difficult to interpret since drug concentrations are fixed arbitrarily. Despite these technical limitations, \textit{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.

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Authors’ addresses: Leonardo K. Basco, Unité de Recherche Paludologique 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,
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