

IN VITRO ACTIVITY OF ANTIMALARIALS AGAINST CLINICAL ISOLATES OF *PLASMODIUM FALCIPARUM* IN YAOUNDE, CAMEROON

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Abstract. The in vitro activity of nine antimalarials was determined against 119 fresh clinical isolates of *Plasmodium falciparum* obtained from symptomatic indigenous patients in Yaounde, Cameroon, using the isotopic semimicrotest. Seventy-four parasites were resistant to chloroquine (mean 50% inhibitory concentration [IC₅₀] 337 nM); 45 were chloroquine-sensitive (mean IC₅₀ 35.6 nM). Twenty-five of 58 chloroquine-resistant parasites were resistant to monodesethylamodiaquine, the biologically active metabolite of amodiaquine. None of the chloroquine-sensitive isolates was resistant to monodesethylamodiaquine (IC₅₀ 17.3 nM). Pyronaridine, quinine, mefloquine, halofantrine, and artemether were highly active against the chloroquine-sensitive and the chloroquine-resistant isolates. Of the 43 isolates tested, 25 were sensitive to both pyrimethamine and cycloguanil, the biologically active metabolite of proguanil. The in vitro responses of chloroquine and monodesethylamodiaquine, chloroquine and quinine, quinine and mefloquine, mefloquine and halofantrine, artemether and mefloquine or halofantrine, and pyrimethamine and cycloguanil were significantly correlated. The present study suggests that chloroquine resistance is highly prevalent in vitro in Yaounde and that the alternative drugs are generally highly active against the chloroquine-resistant parasites.

Malaria is one of the most prevalent parasitic diseases in Cameroon, central Africa. Malaria transmission is seasonal in the northern half of the country, dominated by savanna, and continuous throughout the year in the southern tropical rain forest.¹ A large majority of malaria infections are due to *Plasmodium falciparum*.

Chloroquine-resistant *P. falciparum* was first detected in expatriates in Limbe, a coastal town in southern Cameroon, and in African patients in Yaounde in 1985-1986.^{2,3} During this period, more than 65% of falciparum isolates from Limbe were resistant in vitro to chloroquine.^{4,5} Brasseur and others⁶ found full sensitivity to chloroquine among the isolates obtained in other localities in southern Cameroon in 1985. Since then, several in vitro studies have demonstrated the spread of chloroquine resistance to other parts of the country.^{5,7-12} The proportion of chloroquine-resistant isolates seems to have stabilized at 50-60% in southern Cameroon.^{11,13}

Yaounde, the study site of the present study, is the capital city of Cameroon with an estimated population of 1,000,000. It is situated in the continuous malaria transmission zone in the southern highlands at an average altitude of 760 meters above sea level. The city is surrounded by a dense tropical rain forest. The equatorial climate is characterized by two rainy seasons (March-May, September-November) and two dry seasons (December-February, June-August). Both children and adults are affected by acute uncomplicated malaria, the children being symptomatic approximately two or three times more often than adults (Ringwald P, unpublished data).

Previous epidemiologic studies of drug resistance have analyzed the response of Cameroonian isolates of *P. falciparum* to the standard antimalarials chloroquine, quinine, and mefloquine.⁵⁻⁹ Yaounde is one of the urban sites in which both in vitro tests and in vivo studies in symptomatic patients have been conducted regularly by the Organisation de la Lutte contre les Endémies en Afrique Centrale (OCEAC).^{10,11} The aims of the present study were 1) to determine the in vitro response of fresh clinical isolates to stan-

dard antimalarials chloroquine, monodesethylamodiaquine (the biologically active metabolite of amodiaquine), quinine, pyrimethamine, and cycloguanil (the biologically active metabolite of proguanil), as well as to newer drugs (mefloquine, halofantrine, artemether, and pyronaridine) and 2) to evaluate whether there is a positive correlation between the in vitro responses to different antimalarials. The in vitro data were compared with the clinical efficacy of the drugs in recent clinical trials conducted in Yaounde.

MATERIALS AND METHODS

Parasites. One hundred nineteen fresh clinical isolates of *P. falciparum* were obtained before treatment from symptomatic, African adult and pediatric patients attending the Nlongkak dispensary in Yaounde between April 1994 and March 1995. The patients were questioned about drug intake and screened for self-medication by the urine test of Saker-Solomons.¹⁴ Due to cross-reactivity, the urine assay gives a positive result for chloroquine, quinine, mefloquine, proguanil, cycloguanil, and pyrimethamine. Giemsa-stained thin blood smears were examined for parasite identification, and parasite density was determined against 5,000 erythrocytes. Five to ten milliliters of venous blood were collected in a tube coated with EDTA (Terumo Europe N. V., Leuven, Belgium) after patients' informed consent was obtained. A smaller volume of blood was obtained from young children. Samples with a mono-infection due to *P. falciparum* and a parasite count > 0.2% from patients whose urine test result was negative were used in this study. All drug assays were performed within 5 hr after blood extraction.

Drugs. Chloroquine sulfate was provided by Specia (Paris, France), monodesethylamodiaquine by Parke-Davis (Courbevoie, France), mefloquine hydrochloride by Hoffman-La Roche (Basel, Switzerland), and halofantrine hydrochloride by SmithKline & Beecham (Hertfordshire, United Kingdom). Pyronaridine tetraphosphate was provided by Professor C. Chen (Institute of Parasitic Diseases, Shang-



hai, China). Cycloguanil base was supplied by Imperial Chemical Industries (Hertfordshire, United Kingdom). Quinine hydrochloride and pyrimethamine base were obtained from the Sigma Chemical Company (St. Louis, MO). Artemether was obtained from Rhone-Poulenc-Rorer (Antony, France). Stock solutions of chloroquine, monodesethylamodiaquine, and cycloguanil were prepared in sterile distilled water. Stock solutions of mefloquine, halofantrine, quinine, pyrimethamine, and artemether were prepared in methanol. Two-fold serial dilutions of the drugs were made in sterile distilled water. The final concentration of methanol did not exceed 0.1%, and the solvent alone did not affect the parasite growth. The final concentrations (in two-fold dilutions) ranged from 12.5 to 1,600 nM for chloroquine and quinine, 1.25 to 160 nM for monodesethylamodiaquine and pyronaridine, 2.5 to 400 nM for mefloquine, 0.25 to 32 nM for halofantrine, 0.5 to 64 nM for artemether, and 3.1 to 51,200 nM (in four-fold dilutions) for cycloguanil and pyrimethamine. Each concentration was distributed in triplicate in 24-well tissue culture plates. All isolates were tested against chloroquine. The isolates obtained from adult patients were tested against nine compounds. The isolates obtained from young children were tested against fewer antimalarials due to a smaller volume of blood extracted from these patients.

In vitro drug sensitivity assay. The blood samples were washed three times in RPMI 1640 medium. The erythrocytes were resuspended in the complete RPMI 1640 medium, consisting of 10% human serum (obtained from European blood donors without previous history of malaria), 25 mM HEPES, and 25 mM NaHCO₃, at a hematocrit of 1.5% and an initial parasitemia of 0.2–1.0%. A medium (RPMI 1640) with a low concentration of folic acid and *p*-aminobenzoic acid was used to assess the sensitivity to cycloguanil and pyrimethamine. If the blood sample had a parasitemia > 1.0%, fresh uninfected, type A⁺ erythrocytes were added to adjust the parasitemia to 0.6%.

The isotopic semi-microtest described by Le Bras and Deloron was used in this study.¹⁵ Seven hundred microliters of the suspension of infected erythrocytes were distributed in each well of the 24-well tissue culture plates. The parasites were incubated at 37°C in 5% CO₂ for 18 hr. To assess parasite growth, ³H-hypoxanthine (specific activity 5 Ci/mmol, 1 µCi/well; Amersham, Buckinghamshire, United Kingdom) was added. After an additional 24 hr of incubation, the plates were frozen to terminate the in vitro drug sensitivity assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant®; Amersham) were added. The incorporation of ³H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden).

The 50% inhibitory concentration (IC₅₀) values, defined as the drug concentration corresponding to 50% of the uptake of ³H-hypoxanthine measured in the drug-free control wells, were determined by linear regression analysis of logarithm of concentrations plotted against the logit of growth inhibition. The threshold IC₅₀ values for in vitro resistance to chloroquine, monodesethylamodiaquine, quinine, mefloquine, halofantrine, cycloguanil, and pyrimethamine were

estimated to be > 100 nM, > 60 nM, > 600 nM, > 30 nM, > 6 nM, > 50 nM, and > 100 nM, respectively.^{16–18} The resistance levels of artemether and pyronaridine are still undefined. Data were expressed as geometric mean IC₅₀ values and the 95% confidence intervals. The mean IC₅₀ values of various drugs were compared between the chloroquine-sensitive and the chloroquine-resistant isolates using the Student's *t*-test. Correlation of the logarithmic values of IC₅₀ for different drugs was calculated by a linear regression analysis. Data were analyzed with the Statview software (Abacus Concepts, Inc., Calabasas, CA). Several assays were not interpretable due to bacterial contamination or technical error in the preparation of test plates and were excluded from data analysis.

RESULTS

Seventy-four (62%) of 119 fresh clinical isolates of *P. falciparum* were resistant to chloroquine (geometric mean IC₅₀ 337 nM) (Table 1). The mean IC₅₀ value of 45 chloroquine-sensitive isolates was 35.6 nM. Ninety isolates were tested for their sensitivity to monodesethylamodiaquine. Twenty-five (28%) isolates were resistant in vitro to monodesethylamodiaquine; all of them were also resistant to chloroquine. Of the 65 isolates sensitive to monodesethylamodiaquine, 33 (51%) were chloroquine-resistant. The mean IC₅₀ values for pyronaridine were 5.15 nM and 4.92 nM against the chloroquine-sensitive isolates and the chloroquine-resistant parasites (a total of 103 isolates tested), respectively. Four of 100 isolates were resistant in vitro to quinine (IC₅₀ values 606 nM, 611 nM, 756 nM, and 1,047 nM); two isolates had IC₅₀ values that were very close to the threshold value (IC₅₀ 600 nM). The quinine-resistant isolates were resistant to chloroquine. Ninety-two isolates were tested for sensitivity to mefloquine and halofantrine. Two parasites were resistant in vitro to mefloquine and halofantrine (IC₅₀ 31 nM and 6.6 nM, respectively) or to mefloquine alone (IC₅₀ 36 nM). In both isolates, the IC₅₀ values slightly exceeded the cutoff points (30 nM for mefloquine, 6 nM for halofantrine). Artemether was highly active (IC₅₀ ≤ 11.0 nM) against all 85 isolates tested. Of the 43 isolates tested, 25 (58%) were sensitive to both cycloguanil (mean IC₅₀ 8.79 nM) and pyrimethamine (mean IC₅₀ 16.3 nM) (Table 2). Fourteen and 16 isolates were highly resistant (cycloguanil IC₅₀ > 500 nM, pyrimethamine IC₅₀ > 2,000 nM) to cycloguanil (IC₅₀ 1,030 nM) and pyrimethamine (IC₅₀ 7,710 nM), respectively.

The IC₅₀ values of chloroquine-resistant isolates for monodesethylamodiaquine (53.2 nM) and quinine (239 nM) were significantly higher (*P* < 0.05) than the IC₅₀ values of chloroquine-sensitive isolates (17.3 nM and 108 nM, respectively). The chloroquine-resistant isolates had lower mean IC₅₀ values for artemether (1.61 nM) in comparison with the chloroquine-sensitive isolates (2.37 nM). The activity of mefloquine, halofantrine, and pyronaridine did not differ (*P* > 0.05) between the chloroquine-sensitive isolates and the chloroquine-resistant isolates. The in vitro responses of chloroquine and monodesethylamodiaquine (*r* = 0.817), chloroquine and quinine (*r* = 0.557), quinine and mefloquine (*r* = 0.505), mefloquine and halofantrine (*r* = 0.693), artemether and mefloquine (*r* = 0.627) or halofantrine (*r* = 0.609), and

TABLE 1
In vitro drug sensitivity patterns of *Plasmodium falciparum* parasites isolated in Yaounde, Cameroon in 1994–1995

Drug	Drug susceptibility (nM)*			
	Chloroquine-sensitive (n = 45)		Chloroquine-resistant (n = 74)	
	IC ₅₀	95% confidence limits	IC ₅₀	95% confidence limits
Chloroquine	35.6	30.6–41.5	337	305–372
Monodesethylamodiaquine	17.3	14.0–21.3	53.2	46.2–61.1
Pyronaridine	5.15	4.18–6.35	4.92	4.14–5.86
Quinine	108	87.1–134	239	199–287
Mefloquine	7.38	5.79–9.40	8.47	6.78–10.6
Halofantrine	1.32	1.04–1.66	1.22	1.04–1.44
Artemether	2.37	1.91–2.94	1.61	1.30–2.00

* Values are the geometric mean 50% inhibitory concentration (IC₅₀).

cycloguanil and pyrimethamine ($r = 0.975$) were significantly correlated (Table 3).

DISCUSSION

The in vitro activity of a wide range of reference anti-malarials as well as newer antimalarial agents was determined against fresh isolates of *P. falciparum* obtained in the urban setting in Yaounde, Cameroon. In the previous in vitro studies conducted in Yaounde, it had been shown that about 50–60% of *P. falciparum* were resistant to chloroquine, both in vivo and in vitro.¹¹ A similar proportion of parasites were resistant in vitro to chloroquine in the present study.

Because of the cross-resistance between chloroquine and amodiaquine in highly chloroquine-resistant areas in Asia and the possibility of hematologic and hepatic toxicity of amodiaquine when it is used for chemoprophylaxis, the World Health Organization (WHO) no longer recommends the use of amodiaquine for malaria treatment or prophylaxis.¹⁹ However, recent clinical studies conducted in Africa demonstrate the high efficacy of amodiaquine and lack of serious toxic effects.^{20–22} The in vitro data also suggest the high activity of amodiaquine against most chloroquine-resistant isolates and tend to support the clinical efficacy of amodiaquine in central and West Africa.^{4, 17, 23} Cross-resistance between chloroquine and amodiaquine suggested by these in vitro studies as well as by the present study has so far not been observed in the clinical practice in Yaounde.

The in vitro studies of Brasseur and others^{5, 13} have suggested the presence of a considerable number of quinine-resistant isolates (up to 25%) in southern Cameroon. In recent clinical studies involving a total of 113 patients in Yaounde, 10 cases of therapeutic failure with quinine (oral

or parenteral administration of 25 mg of base/kg/day for 3–5 days) have been reported with a recrudescence of parasitemia on day 14.^{24–27} At least three of 10 treatment failures were ascribed to poor compliance. The other cases of treatment failure were probably not true cases of quinine resistance since the duration of quinine therapy was only three days, instead of the standard seven days, and some of these patients responded to a second quinine treatment given over 3–5 days. The clinical efficacy of quinine is supported by the in vitro data of the present study and those of other studies that suggest high activity of quinine against a large majority of Cameroonian isolates in Yaounde.^{11, 23, 28} The wide discrepancy between the in vitro predictions of Brasseur and others and the high clinical efficacy of quinine is probably due to the low threshold level for in vitro resistance to quinine set by these investigators (IC₅₀ > 300 nM).

Sulfadoxine-pyrimethamine (for treatment) and pyrimethamine alone (for prophylaxis in pregnant women) have been widely used in Cameroon since the late 1980s. Cameroon has recorded the highest volume of consumption of these two drugs in francophone Africa. A clinical trial conducted in the late 1980s in Yaounde showed the full efficacy of sulfadoxine-pyrimethamine.²⁶ The first case of resistance to this drug in Cameroon was reported in 1987 in a non-immune traveler on chemoprophylaxis with sulfadoxine-pyrimethamine, who failed to respond to the same drug combination given at the therapeutic dose.²⁹ Our in vitro data suggest higher activity of pyrimethamine against 58% of Cameroonian isolates. The presence of highly pyrimethamine-resistant isolates (37% of the isolates tested) seems to reflect the drug pressure exerted in the country for almost a decade. More recent clinical evaluation of the efficacy of

TABLE 2
In vitro susceptibility of Cameroonian isolates of *Plasmodium falciparum* to pyrimethamine and cycloguanil

Drug	In vitro response (nM)*					
	Susceptible†		Intermediate†		Resistant†	
	IC ₅₀	95% confidence limits	IC ₅₀	Range	IC ₅₀	95% confidence limits
Pyrimethamine	16.3	11.8–22.7	409	179–936	7,710	6,110–9,750
	(n = 25)		(n = 2)		(n = 16)	
Cycloguanil	8.79	6.73–11.5	144	56.2–366	1,030	778–1,360
	(n = 25)		(n = 4)		(n = 14)	

* Values are the geometric mean 50% inhibitory concentration (IC₅₀).

† Based on the previous study on African isolates,¹⁷ in vitro response was defined as susceptible (IC₅₀ < 100 nM for pyrimethamine, < 50 nM for cycloguanil), intermediate (IC₅₀ 100–2,000 nM for pyrimethamine, 50–500 nM for cycloguanil), and resistant (IC₅₀ > 2,000 nM for pyrimethamine, > 500 nM for cycloguanil).

TABLE 3

Correlation of in vitro responses of antimalarial drugs against Cameroonian isolates of *Plasmodium falciparum**

Drug pairs		Correlation coefficient	Probability
Chloroquine	Monodesethylamodiaquine	0.817	<0.01
Chloroquine	Pyronaridine	0.047	NS
Chloroquine	Quinine	0.557	<0.01
Chloroquine	Mefloquine	0.264	0.02
Chloroquine	Halofantrine	0.064	NS
Chloroquine	Artemether	-0.083	NS
Quinine	Mefloquine	0.505	<0.01
Quinine	Halofantrine	0.373	<0.01
Mefloquine	Halofantrine	0.693	<0.01
Mefloquine	Artemether	0.627	<0.01
Halofantrine	Artemether	0.609	<0.01
Cycloguanil	Pyrimethamine	0.975	<0.01

* The correlation coefficient (r) was calculated by linear regression analysis of logarithmic 50% inhibitory concentration values. Probability refers to the significance level of the test ($P < 0.05$). NS = nonsignificant.

sulfadoxine-pyrimethamine and regular epidemiologic monitoring are needed to confirm the in vitro findings.

Proguanil, in combination with chloroquine, is one of the recommended chemoprophylactic regimens for indigenous pregnant women and nonimmune travelers and residents. Chloroquine-proguanil is well-tolerated and generally effective for long-term use. Clinical trials in Yaounde have shown that most nonimmune residents on chloroquine-proguanil were protected.^{30,31} As with pyrimethamine, the in vivo response to chloroquine-proguanil should be monitored periodically to ensure its efficacy. The high correlation of the in vitro response to pyrimethamine and cycloguanil was expected since both drugs act on the same molecular target, dihydrofolate reductase-thymidylate synthase.

Of the new antimalarials evaluated in our study, pyronaridine, artemether, mefloquine, and halofantrine displayed a high in vitro activity. Pyronaridine and artemether have been shown to be active in vitro against African isolates in previous studies.^{32,33} They have undergone clinical evaluation in Yaounde, and both drugs were shown to be highly effective (Louis F, unpublished data).³⁴ The high efficacy of halofantrine to treat malaria in symptomatic African adult patients has been demonstrated in a clinical trial conducted in Yaounde in 1990-1991.³⁵ Our own experience in successfully treating recrudescing adult and pediatric cases with halofantrine after correct chloroquine therapy lends further support to its high clinical efficacy. Mefloquine is not available in Cameroon, except in a form of the triple combination, mefloquine-sulfadoxine-pyrimethamine, that has not been widely used in a community or national level. The efficacy of this combination and that of mefloquine alone (15 mg/kg in a single dose) has been demonstrated in Yaounde (Ringwald P, unpublished data).³⁶ Based on slow parasite clearance in healthy asymptomatic schoolchildren, Brasseur and others³⁷ have concluded that RII-RIII levels of mefloquine resistance exist in northern Cameroon. However, according to the inclusion criteria of the vivo test defined by the WHO, asymptomatic parasite carriers are not suitable subjects for clinical trials that aim to evaluate clinical efficacy.

Amodiaquine is becoming one of the first-line drug in Yaounde and in some other parts of Cameroon. Regular monitoring of the clinical efficacy of amodiaquine and its

correct therapeutic use are therefore needed to ensure its maximal utility. Because of the limited availability of the newer drugs in the country, the positive in vitro correlation between mefloquine and halofantrine does not have any clinical repercussion at present. The in vitro and in vivo surveillance of pyrimethamine and cycloguanil is necessary as sulfadoxine-pyrimethamine becomes widely used for the treatment of chloroquine-resistant cases. The high in vitro activity of the newer antimalarial drugs assessed in the present study is supported by recent clinical trials in the same urban setting.³⁴⁻³⁶ In vitro drug assays, in conjunction with clinical studies, are valuable tools in monitoring the status of the rapidly changing epidemiology of drug resistance and in guiding the national antimalarial drug policy.

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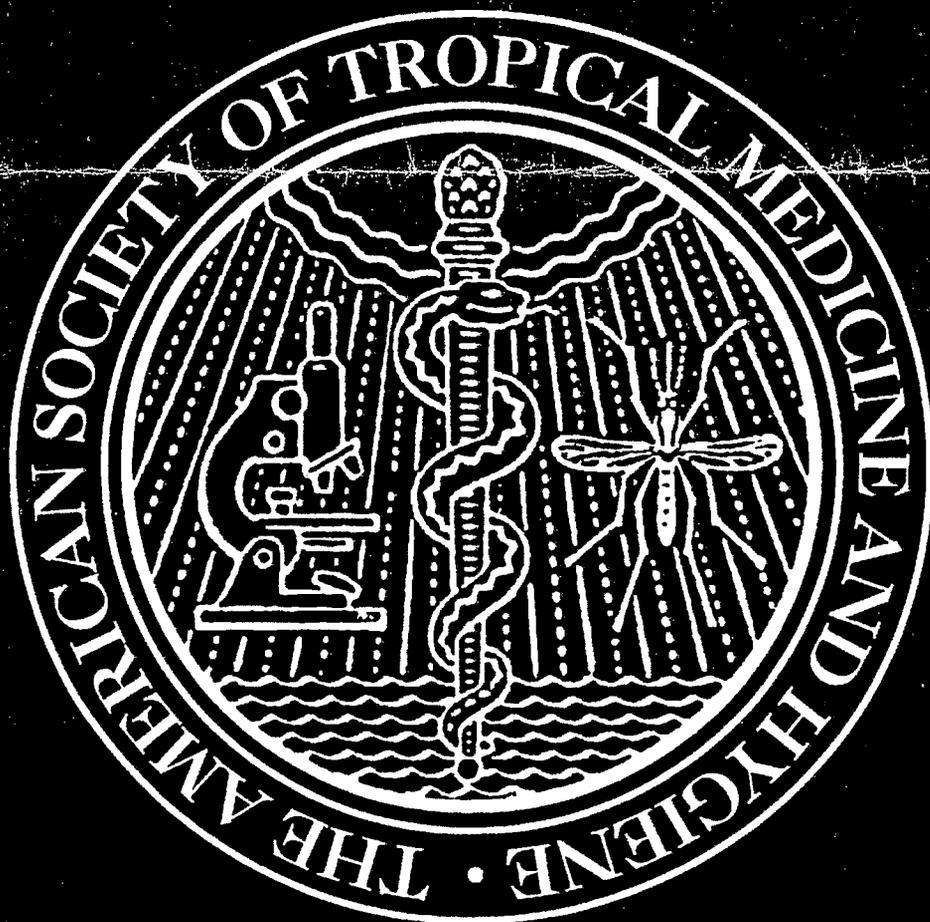
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