

IN VITRO ACTIVITY OF ARTEMETHER AGAINST AFRICAN ISOLATES (SENEGAL) OF *PLASMODIUM FALCIPARUM* IN COMPARISON WITH STANDARD ANTIMALARIAL DRUGS

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Abstract. The in vitro activity of artemether against 56 African isolates of *Plasmodium falciparum* from Senegal was evaluated using an isotope-based drug susceptibility semi-microtest. The 50% inhibitory concentration (IC₅₀) values for artemether were in a narrow range from 0.8 to 15.2 nM (mean IC₅₀ = 3.43 nM) and the 95% confidence interval (CI) was 2.50–4.36 nM. Artemether was equally effective on chloroquine-sensitive and chloroquine-resistant isolates (mean IC₅₀ = 346 nM, 95% CI = 2.08–4.84 nM versus mean IC₅₀ = 2.80 nM, 95% CI = 2.00–3.60 nM). There was a significant positive correlation between responses to artemether and mefloquine ($r^2 = 0.36$, $P < 0.001$), artemether and quinine ($r^2 = 0.085$, $P < 0.05$), artemether and halofantrine ($r^2 = 0.075$, $P < 0.05$), quinine and mefloquine ($r^2 = 0.205$, $P < 0.01$), quinine and halofantrine ($r^2 = 0.124$, $P < 0.05$), and mefloquine and halofantrine ($r^2 = 0.801$, $P < 0.001$). A positive correlation between these drugs suggests in vitro cross-resistance or at least in vitro cross-susceptibility.

In the absence of effective and practical preventive measures, the only current options for reducing the morbidity and mortality of malaria especially in Africa are chemoprophylaxis and chemotherapy. For this reason, the increasing prevalence of strains of *Plasmodium falciparum* resistant to chloroquine and other antimalarial drugs poses a serious problem for control of malaria.¹ There is an urgent need to find and develop alternative drugs against chloroquine-resistant *P. falciparum*.

One group of alternative antimalarial drugs comprises artemisinin (qinghaosu) and its derivatives. Artemisinin is a traditional Chinese medicinal herb derived from *Artemisia annua*. It has a sesquiterpene lactone with a peroxide bridge and antimalarial activity has been attributed to the peroxide moiety² by a mechanism involving production of free radicals that damage the parasite membrane.³ Artemether (an oil-soluble artemisinin derivative) has shown effectiveness against chloroquine-resistant strains of *P. falciparum* in China and Thailand.^{4,5} However, little information is available concerning the in vitro activity of artemisinin and its derivatives against isolates of *P. falciparum* from Africa other than reference strains.^{6,7}

The two-fold aim of this in vitro study was to evaluate the activity of artemether against fresh isolates of *P. falciparum* from Senegal, and to analyze cross-susceptibility between artemether, chloroquine, quinine, mefloquine, and halofantrine.

MATERIALS AND METHODS

Isolates of *P. falciparum*. All programs were reviewed and approved by the Conseil de Perfectionnement of the Institut de Dakar, which is presided over by the Senegalese Health Secretary. Informed oral consent was obtained from the patients and/or their parents before collection of blood samples. Only oral consent was obtained because only 5% of the people in Dielmo, Ndiop, and Toubakouta can read and write. Between October 1995 and January 1996, 56 fresh *P. falciparum* isolates were prepared from samples obtained after oral consent in Dielmo, Ndiop, and Toubakouta (280 km southeast of Dakar) in

the Fatick region of Senegal. Patients from Toubakouta were recruited at the public center while patients from Dielmo and Ndiop were recruited at home by daily active case detection during a longitudinal study of the mechanisms of protective immunity to malaria.^{8,9} If the parasite density was greater than 2,500 rings/ μ l of blood for patients in Toubakouta and Ndiop (seasonal transmission) and greater than 15,000 rings/ μ l for patients in Dielmo (continuous transmission), then venous blood was collected before treatment in Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ) and transported at 4°C to our laboratory in Marseille within 84 hr. Thin blood smears were stained using an RAL® kit (Reactifs RAL, Paris, France) and examined to determine parasite density and confirm monoinfection by *P. falciparum*. Samples with parasitemias ranging from 0.1% to 8.0% were used to test drug sensitivity. Parasitized erythrocytes were washed three times in RPMI 1640 medium (Gibco BRL, Paisley, United Kingdom). If parasitemia exceeded 0.8%, infected erythrocytes were diluted to 0.5–0.8% with uninfected erythrocytes and resuspended in culture medium to a hematocrit of 1.5%. Parasite growth was assessed after 42 hr of incubation before reinvasion. Susceptibility to chloroquine, quinine, mefloquine, halofantrine, and artemether was determined after suspension of the erythrocytes in RPMI 1640 medium supplemented with 10% human serum (pooled from different A⁺ or AB⁺ nonimmune donors who were from outside the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO₃.

Plasma measurements of the relevant antimalarials were evaluated by high-performance liquid chromatography. Traces of chloroquine were found in the plasma of 70% of the patients, but there was no evidence that traces of antimalarials in infected erythrocytes after three washings of the blood in RPMI 1640 medium affected the in vitro assays.

Drugs. Chloroquine diphosphate and quinine hydrochloride were obtained from Sigma Chemicals (St. Louis, MO), mefloquine hydrochloride from Roche (Paris, France), halofantrine hydrochloride from Smith-Kline Beecham (Hertfordshire, United Kingdom), and artemether from Rhone Poulenc Rorer (Antony, France). Stock solutions were pre-



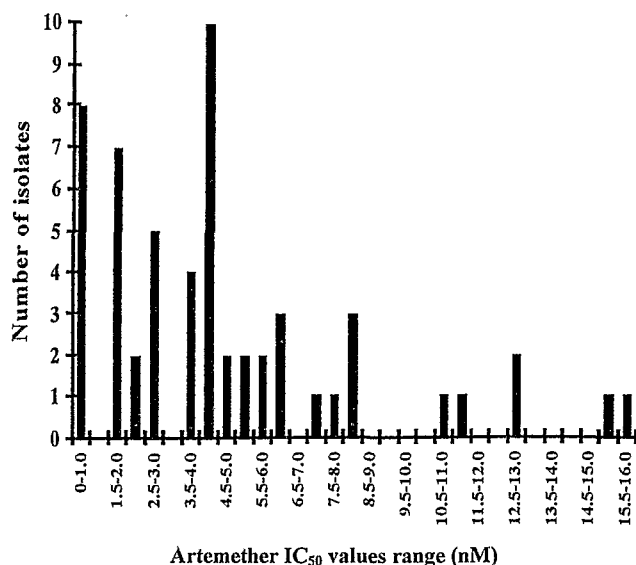


FIGURE 1. In vitro response of 56 isolates of *Plasmodium falciparum* from Senegal to artemisinin. IC_{50} = 50% inhibitory concentration in nanomoles/liter.

pared in sterile distilled water for chloroquine diphosphate and in methanol for the other antimalarial agents. Previous studies in our laboratory indicated that methanol had no cytotoxicity on parasite growth at the lowest dilution (methanol/water) used with the highest final concentration of antimalarial. There was no evidence of precipitation of antimalarials when dilutions were made in water. Two-fold serial dilutions were prepared in sterile distilled water and distributed in triplicate into 24-well flat-bottomed plates (no. 13124; Nunc, Roskilde, Denmark). Final concentrations, which ranged from 25 to 3,200 nM for chloroquine, 50 to 3,200 nM for quinine, 2.5 to 400 nM for mefloquine, 0.25 to 32 nM for halofantrine, and 0.8 to 100 nM for artemether, were prepared in triplicate in 24-well plates.

The chloroquine-susceptible *P. falciparum* D6 clone (Sierra Leone) and the chloroquine-resistant W2 clone (Indochina) were used as references to test each batch of plates. Plate batches with an in vitro response 15–20% below or above the mean of the relevant antimalarials were eliminated. Reference clones were maintained in continuous culture and were synchronized twice with sorbitol.

In vitro assay. The in vitro semi-microtest used in the present study has been previously described by Le Bras and Deloron.¹⁰ The suspension of parasitized erythrocytes (750 μ l/well) was distributed in 24-well plates pre-dosed with antimalarial agents. Plates were incubated for 42 hr at 37°C in an atmosphere of 10% O_2 , 6% CO_2 , 84% N_2 , and a humidity of 95%. These had been previously established as optimum conditions in our laboratory. Parasite growth was assessed after 18 hr by adding 1 μ Ci of 3H -hypoxanthine with a specific activity 14.1 Ci/mmol (New England Nuclear Products, Dreiech, Germany) to each well. Immediately after incubation, the plates were frozen and thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter[®] CF/B; Packard Instrument Co., Meriden, CT) and washed using a cell harvester (FilterMate[®] Cell Harvester, Packard Instrument Co.). Filter microplates were dried and 100

TABLE 1

In vitro response of 50 African isolates of *Plasmodium falciparum* from Senegal to chloroquine and artemether*

Drug	Chloroquine-sensitive isolates (n = 32)		Chloroquine-resistant isolates (n = 18)	
	IC_{50} (nM)	95% confidence intervals	IC_{50} (nM)	95% confidence intervals
Chloroquine	41.0	32.4–49.6	179.3	132.4–226.2
Artemether	3.46	2.08–4.84	2.8	2.01–3.59

* Values are geometric mean 50% inhibitory concentrations (IC_{50}) in nanomoles/liter.

μ l of scintillation cocktail (Microscint[®] O; Packard Instrument Co.) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count[®]; Packard Instrument Co.).

The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration corresponding to 50% of the uptake of 3H -hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves. Isolates were considered chloroquine-resistant if the IC_{50} was greater than 100 nM. Cut-off values for resistance to quinine, mefloquine, and halofantrine were 500 nM, 20 nM, and 5 nM, respectively. The in vitro threshold values for antimalarials have been defined statistically (> 2 SD above the mean). In vitro resistance to chloroquine was evaluated statistically and by correlation between in vitro response and therapeutic effectiveness. The cut-off value for artemether resistance was not determined because until now only in vitro susceptibility of reference clones or of a restricted number of isolates had been determined. These data were not enough to evaluate statistically the in vitro resistance cut-off value. Data were expressed as the geometric mean IC_{50} and 95% confidence intervals (95 CIs) were calculated. The unpaired *t*-test was used to compare IC_{50} values from chloroquine-susceptible and chloroquine-resistant isolates. Assessment of standard cross-resistance of other antimalarials with artemether was estimated by the Pearson correlation coefficient (*r*) and the coefficient of determination (*r*²).

RESULTS

The IC_{50} values for artemether were in a narrow range from 0.8 to 15.2 nM (mean IC_{50} = 3.43 nM) and the 95% CI was 2.50–4.36 nM (Figure 1). Based on our criterion (IC_{50} > 100 nM), 18 of the 56 fresh isolates of *P. falciparum* studied were considered as chloroquine-resistant (mean IC_{50} = 179 nM, 95% CI = 132–226 nM) (Table 1). As shown in Table 1, the IC_{50} values for artemether obtained with chloroquine-sensitive isolates and chloroquine-resistant isolates were similar (mean IC_{50} = 3.46 nM, 95% CI = 2.08–4.84 nM versus mean IC_{50} = 2.80 nM, 95% CI = 2.00–3.60 nM).

A significant positive correlation was observed between the responses to artemether and mefloquine (r^2 = 0.360, P < 0.001), artemether and quinine (r^2 = 0.085, P < 0.05), artemether and halofantrine (r^2 = 0.075, P < 0.05), quinine and mefloquine (r^2 = 0.205, P < 0.01), quinine and halofantrine (r^2 = 0.124, P < 0.05), and mefloquine and halofantrine (r^2 = 0.801, P < 0.001) (Table 2). A significant negative correlation was observed between the responses to chloroquine and mefloquine (r^2 = 0.137, P < 0.05).

TABLE 2

Correlation between in vitro responses of African isolates of *Plasmodium falciparum* from Senegal to artemether and standard antimalarial drugs

Drug pair		Number of isolates	Correlation coefficient r	Coefficient of determination r^2	P
Artemether	Chloroquine	50	-0.172	0.030	>0.1
Artemether	Quinine	52	0.291	0.085	<0.05
Artemether	Mefloquine	31	0.600	0.360	<0.001
Artemether	Halofantrine	50	0.273	0.075	<0.05
Chloroquine	Quinine	49	0.008	<0.001	>0.1
Chloroquine	Mefloquine	31	-0.370	0.137	<0.05
Chloroquine	Halofantrine	48	-0.143	0.020	>0.1
Quinine	Mefloquine	31	0.453	0.205	<0.01
Quinine	Halofantrine	49	0.352	0.124	<0.05
Mefloquine	Halofantrine	30	0.895	0.801	<0.001

DISCUSSION

The cut-off value for resistance to artemether remains unclear. Based on statistical calculations using the present data (mean $IC_{50} + 2$ SD), the cut-off value for resistance to artemether is an IC_{50} value greater than 10.5 nM. This is consistent with a value of 10.0 nM previously estimated for resistance of African isolates to artemether (Le Bras J, unpublished data). However, artemether has been shown to be effective in vivo and no therapeutic failure of artemether has been reported. The six isolates in this study with IC_{50} values greater than 10.5 nM were not considered resistant. Accurate determination of the IC_{50} cut-off value for resistance will require correlation between responses observed in vitro and results obtained after chemotherapy in vivo.

In the present study, the in vitro response of the chloroquine-susceptible and chloroquine-resistant isolates to artemether did not differ significantly. This finding is in contrast with previous studies in Central and West Africa¹¹ and in Cameroon,¹² which showed that susceptibility to artemether was inversely related to susceptibility to chloroquine.

Also in contrast with previous findings indicating negative^{6, 11} or weakly positive⁷ correlations between responses to artemisinin and chloroquine, the present study showed no significant correlation. On the other hand, our data did suggest a positive correlation between in vitro responses to artemether, mefloquine, quinine, and halofantrine. Nevertheless, the coefficient of determination (r^2) values for the correlations between artemether and the aminoalcohols are minimal to medium: 0.075 for artemether/halofantrine, 0.085 for artemether/quinine, and 0.36 for artemether/mefloquine. Although these positive correlations are in agreement with previous reports in Africa,⁶ it should be emphasized that a positive correlation in vitro is not necessarily predictive of cross-resistance in vivo. In clinical practice, halofantrine seems to be effective against mefloquine-resistant isolates¹³ despite a positive correlation between the IC_{50} values of halofantrine and mefloquine in 80 of 100 isolates.¹⁴ However, reduced in vitro susceptibility to mefloquine and halofantrine¹⁵ and treatment failures with halofantrine¹⁶ and mefloquine,¹⁷ despite therapeutically effective plasma concentrations, have been reported in west African patients living in areas where halofantrine and mefloquine have not been used extensively (in comparison with

quinine, which has been widely used in these areas). Cross-resistance may explain failures with mefloquine and halofantrine. A study prior to the use of mefloquine in Cameroon showed a correlation between mefloquine and quinine resistance.¹⁸ This finding could indicate that some *P. falciparum* isolates are innately less susceptible to mefloquine and halofantrine or that there is cross-resistance between these aminoalcohols.

Since the mechanisms underlying the antimalarial action of artemether, mefloquine, quinine, and halofantrine are still unclear, one can only speculate about the positive, even minor, correlation between responses to artemether and aminoalcohols (especially mefloquine). Several findings suggest that heme may be the common link between these antimalarials. Indeed, heme or heme polymerase in food vacuole has been reported to be the specific target of aminoalcohols.^{19, 20} Based on their data, Meshnick and others²¹ attributed antimalarial action to a free-radical-generating²² adduct formed between artemisinin and hemin produced by the parasite-induced breakdown of hemoglobin in the erythrocyte.²³ Artemisinin enhances hemin-mediated inhibition of membrane-bound enzymes via oxidation of thiol groups on the enzymes.²⁴ Another possible target for artemisinin and mefloquine is membrane phospholipids.^{25, 26} The interaction between the erythrocyte membrane and artemisinin may involve the formation of a covalent linkage with the peroxide bridge, leading to disruption of membrane structure.²⁷

The correlation between quinine, mefloquine, and halofantrine may be explained by molecular biology. Although the genetic basis of resistance to various antimalarial drugs has not yet been established, it has been reported that the *P. falciparum* multidrug resistance (*Pfmdr1*) gene and its protein product were amplified and overexpressed in chloroquine-resistant cloned isolates subjected to mefloquine selection pressure.²⁸ The same investigators also demonstrated decreased resistance to chloroquine, as well as increased resistance to quinine and halofantrine.

In Thailand, where *P. falciparum* is highly resistant to chloroquine and increasingly resistant to quinine and even mefloquine,²⁹ Luxemburger and others³⁰ showed that possible oral artesunate at standard doses was superior to intravenous quinine for treatment of uncomplicated multidrug-resistant malaria in patients at high risk of developing life-threatening complications. Several recent studies have confirmed that the combination of artemisinin-related compounds with mefloquine is highly effective even against mefloquine-resistant *P. falciparum*.^{4, 31, 32}

Because of its effectiveness against chloroquine-resistant *P. falciparum* malaria, artemether can be considered as a treatment of choice for multidrug-resistant malaria. In addition to effectiveness, two other advantages of artemether treatment are simplicity to administer and good tolerance with no evidence of local toxicity and detectable adverse systemic effects.^{33, 34} However, if confirmed in vivo, the correlation, even if minor, observed in the present in vitro study between responses of *P. falciparum* isolates to artemether, quinine, mefloquine, and halofantrine needs investigation. Nevertheless, for the time being, this may not limit the usefulness of artemether as a replacement drug for quinine in Africa.

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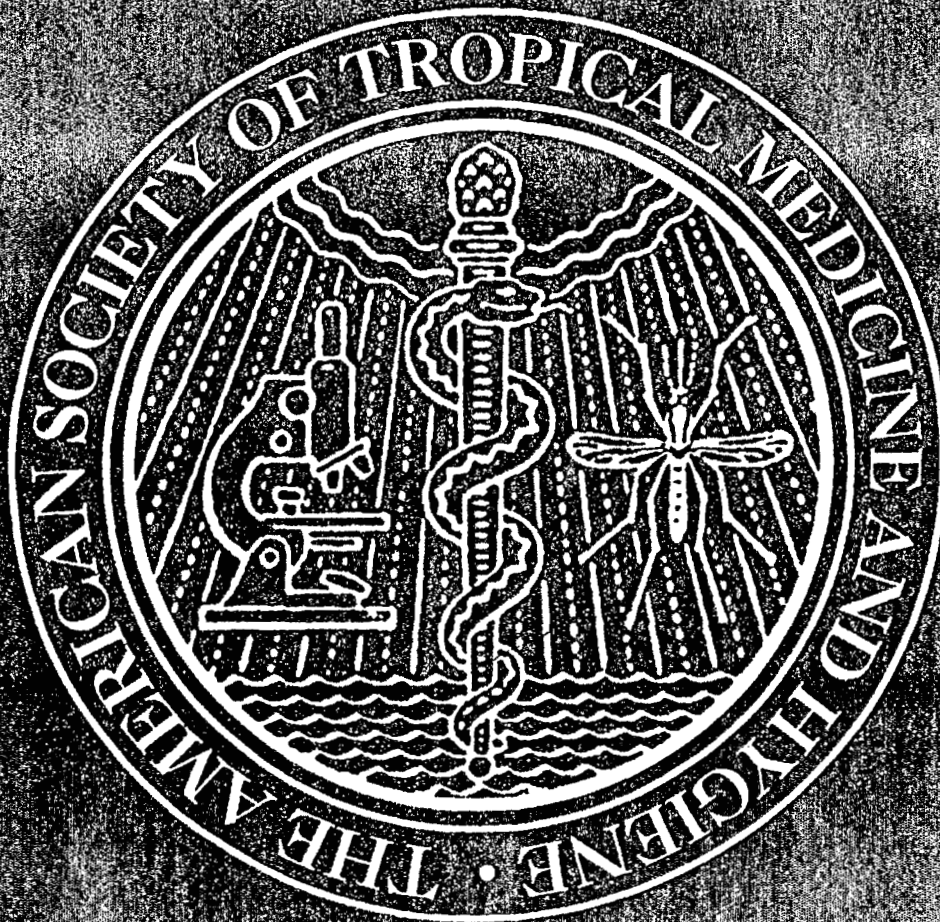
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