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Am. J. Trop. Med. Hyg., 61(2), 1999, pp. 187–192

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

PASCAL RINGWALD, JEAN BICKII, AND LEONARDO K. BASCO

Institut de Recherche pour le Développement (IRD, formerly ORSTOM) and Laboratoire de Recherches sur le Paludisme, Laboratoire Associé Francophone 302, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé, Cameroon

Abstract. The *in vitro* activities of dihydroartemisinin (the biologically active metabolite of artemisinin derivatives), chloroquine, monodesethylamodiaquine (the biologically active metabolite of amodiaquine), quinine, mefloquine, halofantrine, and pyrimethamine were assessed in 65 African isolates of *Plasmodium falciparum* from Yaoundé, Cameroon using an isotopic microtest. The 50% inhibitory concentration (IC_{50}) values for dihydroartemisinin were within a narrow range from 0.25 to 4.56 nM, with a geometric mean of 1.11 nM (95% confidence interval = 0.96–1.28 nM). Dihydroartemisinin was equally active ($P > 0.05$) against the chloroquine-sensitive isolates (geometric mean IC_{50} = 1.25 nM, 95% confidence interval = 0.99–1.57 nM) and the chloroquine-resistant isolates (geometric mean IC_{50} = 0.979 nM, 95% confidence interval = 0.816–1.18 nM). A significant positive correlation was observed between the responses to dihydroartemisinin and mefloquine (r = 0.662) or halofantrine (r = 0.284), suggesting *in vitro* cross-resistance. There was no correlation between the responses to dihydroartemisinin and other antimalarial drugs.

Artemisinin (qinghaosu) has been used for centuries in traditional Chinese medicine for antimalarial treatment.[1] In 1972, Chinese scientists isolated the active principle from the plant *Artemisia annua*.[2] Artemisinin is a sesquiterpene lactone characterized by the presence of an endoperoxide that is associated with a potent antimalarial activity. Because artemisinin is chemically unstable and poorly soluble in water or oil, the carbonyl group at C-10 of the parent compound was reduced to obtain dihydroartemisinin. Several derivatives have been developed by adding an ether, ester, or other substituents to the hydroxyl group of dihydroartemisinin. These semi-synthetic derivatives include the water-soluble derivatives, sodium artesunate and artelinic acid, and the oil-soluble derivatives, artemether and arteether. With the exception of arteether and artelinic acid, these compounds are used to treat malarial infections in endemic countries, mainly in Southeast Asia and, to a lesser extent, in Africa and South America.

Artemisinin derivatives are available in different formulations for oral, parenteral, and rectal administration. Clinical studies have shown that artemisinin derivatives are highly potent, rapidly acting, and well-tolerated blood schizontocides, resulting in shorter parasite clearance times than other antimalarial drugs.[3,4] Artemisinin derivatives are highly effective against *Plasmodium falciparum* isolates that are resistant to other drugs. These derivatives are indicated for the emergency treatment of severe and complicated falciparum malaria by parenteral administration and for the oral treatment of uncomplicated multidrug-resistant malaria.[5] So far, resistance to artemisinin derivatives has not been reported in clinical studies. However, because of the high recrudescence rate when used as monotherapy, many current therapeutic regimens used in Southeast Asia include a combination of one of the artemisinin derivatives and mefloquine.[6–9] The combination therapy with other drugs (lumefantrine, tetracycline, pyrimethamine, desferrioxamine) is under evaluation.[10–13]

Although pharmacokinetic data are incomplete, it has been established that all currently available artemisinin derivatives are metabolized rapidly to dihydroartemisinin, the biologically active main human metabolite.[14–17] In the case of sodium artesunate, the aqueous solution that is reconstituted by dissolving the anhydrous powder of the drug in 5% dextrose solution just before parenteral administration is known to hydrolyze the compound rapidly into dihydroartemisinin.[18] Initial *in vitro* studies on laboratory-adapted clones of *P. falciparum* have shown that dihydroartemisinin is more active than the other artemisinin derivatives.[19] Based on the observation that dihydroartemisinin is a highly active metabolite of artemisinin derivatives and that it is well absorbed by oral administration, Chinese investigators have developed oral dihydroartemisinin for clinical use. Dihydroartemisinin is not commercially available outside China. Results of the recent clinical study in Thailand showed the efficacy of oral dihydroartemisinin, with a cure rate of 90% in 49 patients treated for 7 days.[20]

Despite the absence of official therapeutic guidelines for the use of these derivatives, artemether and artesunate have been commercially available in Cameroon since 1997. We have determined the *in vitro* activity of artemether and artesunate in our previous studies.[21,22] In the present study, we assessed the *in vitro* response of fresh clinical isolates to dihydroartemisinin to 1) establish the baseline sensitivity level of dihydroartemisinin in Cameroon, 2) compare its *in vitro* activity with that of other standard antimalarial drugs, and 3) evaluate the *in vitro* cross-resistance patterns.

MATERIALS AND METHODS

Parasites. Sixty-five clinical isolates of *P. falciparum* were obtained before treatment from symptomatic, African patients attending the Nlongkak dispensary in Yaoundé between 1997 and 1998. The patients were screened for self-medication by the urine test of Saker-Solomons, which was developed for the detection of chloroquine but also detects other 4-aminoquinolines, aminoalcohols, and antifolate drugs due to cross-reaction.[23] Giemsa-stained thin blood smears were examined for *Plasmodium* species identification, and parasite density was determined against 5,000 erythrocytes. Venous blood (10 ml) was collected in a tube coated with EDTA (Terumo Europe N. V., Leuven, Belgium) after patients' informed consent was obtained. Blood sam-

ples with a monoinfection due to *P. falciparum* and a parasite density > 0.2% from patients whose urine test result was negative were used in this study. *In vitro* assays were performed within 4 hr after blood extraction. The patients were treated with amodiaquine, sulfadoxine-pyrimethamine, or quinine. This study was approved by the Cameroonian National Ethics Committee.

Drugs. Antimalarial drugs used in this study were obtained from the following sources: chloroquine diphosphate, quinine hydrochloride, and pyrimethamine base (Sigma Chemical Company, St. Louis, MO); monodesethylamodiaquine hydrochloride (the biologically active human metabolite of amodiaquine) and dihydroartemisinin (Sapec Fine Chemicals, Lugano, Switzerland) through the courtesy of Dr. Piero Olliaro (Tropical Diseases Research, World Health Organization, Geneva, Switzerland); mefloquine hydrochloride (Hoffmann-La Roche, Basel, Switzerland); and halofantrine hydrochloride, SmithKline Beecham (Hertfordshire, United Kingdom). Stock solutions of chloroquine and monodesethylamodiaquine were prepared in sterile distilled water. Stock solutions of quinine, mefloquine, halofantrine, and dihydroartemisinin were prepared in methanol. The stock solution of pyrimethamine was prepared in ethanol. Two-fold (4-fold for pyrimethamine) serial dilutions of the stock solutions were made in distilled water. The final concentrations ranged from 25 to 1,600 nM for chloroquine and quinine, 1.25–160 nM for monodesethylamodiaquine, 2.5–400 nM for mefloquine, 0.25–32 nM for halofantrine, 0.5–64 nM for dihydroartemisinin, and 0.39–51,200 nM for pyrimethamine. Each concentration was distributed in triplicate in 96-well tissue culture plates.

***In vitro* assay.** The venous 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 (from non-immune European donors without previous history of malaria), 25 mM HEPES buffer, and 25 mM sodium bicarbonate at a hematocrit of 1.5 % and an initial parasitemia of 0.2–1.0%. The *p*-aminobenzoic acid- and folic acid-free RPMI 1640 medium was used to assess the *in vitro* sensitivity to 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 microtest developed by Desjardins and others was used in this study.[24] The suspension of infected erythrocytes (200 µl) was distributed in each well of the 96-well tissue culture plates. The parasites were incubated at 37°C in 5% CO_2 for 18 hr. To assess parasite growth, ^{3}H-hypoxanthine (specific activity = 16.3 Ci/mmol, 1 µCi/well; Amersham, Buckinghamshire, United Kingdom) was added. After an additional 24 hr of incubation (48 hr for pyrimethamine), the plates were frozen to terminate the *in vitro* 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 ^{3}H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden).

Data analysis. The 50% inhibitory concentration (IC_{50}) values, defined as the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthine measured in the drug-free control wells, were determined by nonlinear regression analysis of logarithm of concentrations plotted against growth inhibition. Prism™ software (GraphPad Software, Inc., San Diego, CA) was used to obtain the best-fitting sigmoid curve, and the IC_{50} values were calculated using the logistic equation. The threshold IC_{50} values for *in vitro* resistance to chloroquine, monodesethylamodiaquine, quinine, mefloquine, and halofantrine were estimated to be > 100 nM, > 60 nM, > 800 nM, > 30 nM, and > 6 nM, respectively.[21] The resistance levels of pyrimethamine (for a 72-hr incubation) and dihydroartemisinin are still undefined.

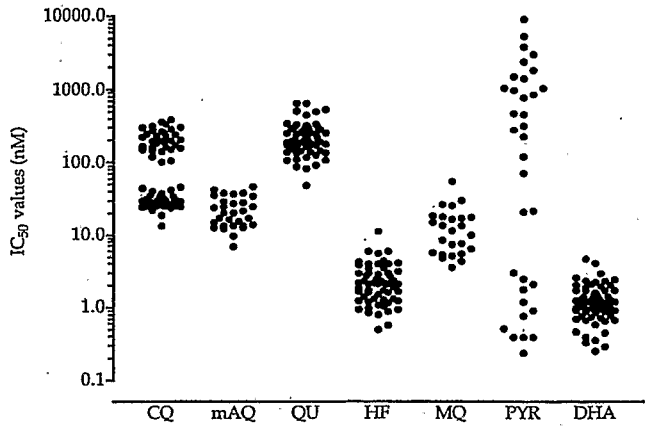

FIGURE 1. Distribution of the 50% inhibitory concentrations (IC_{50}s) in nanomoles of *Plasmodium falciparum* isolates from Cameroon for chloroquine (CQ), monodesethylamodiaquine (mAQ), quinine (QU), halofantrine (HF), mefloquine (MQ), pyrimethamine (PYR), and dihydroartemisinin (DHA).

Data were expressed as geometric mean IC_{50} values and the 95% confidence intervals. The mean logarithmic IC_{50} values of various drugs were compared between the chloroquine-sensitive and the chloroquine-resistant parasites using the Student's unpaired *t*-test. Correlation of the IC_{50} values for different drugs was calculated by Spearman's rank-order correlation test. The significance level was fixed at 0.05. Data were analyzed with the Statview software (Abacus Concepts, Inc., Calabasas, CA).

RESULTS

The *in vitro* activities of chloroquine, monodesethylamodiaquine, quinine, mefloquine, halofantrine, pyrimethamine, and dihydroartemisinin were assessed in 65, 30, 60, 25, 58, 34, and 65 isolates, respectively. Dihydroartemisinin was the most potent drug (geometric mean IC_{50} = 1.11 nM, 95% confidence interval = 0.96–1.28 nM, range = 0.25–4.56 nM, n = 65) among the compounds tested in this study (Figure 1). Dihydroartemisinin was almost twice as active as halofantrine (geometric mean IC_{50} = 1.99 nM, 95% confidence interval = 1.70–2.34 nM) and 10 times more potent than mefloquine (geometric mean IC_{50} = 10.8 nM, 95% confidence interval = 8.12–14.5 nM). The *in vitro* activities of the antimalarial drugs and the number of chloroquine-sensitive and chloroquine-resistant isolates tested for each drug are summarized in Table 1. Monodesethylamodiaquine and quinine were more active ($P < 0.05$) against the chloro-

TABLE 1

In vitro response of chloroquine-sensitive and chloroquine-resistant Cameroonian isolates of *Plasmodium falciparum**

Drug	Chloroquine-sensitive isolates			Chloroquine-resistant isolates			
	n	IC_{50}	95% confidence interval	n	IC_{50}	95% confidence interval	*P*
Chloroquine	33	29.3	26.0–32.9	32	209	185–235	<0.05
Monodesethylamodiaquine	13	13.2	11.2–15.5	17	27.5	23.1–32.6	<0.05
Quinine	30	175	141–217	30	236	204–273	<0.05
Mefloquine	14	13.4	8.9–20.2	11	8.24	5.44–12.5	NS
Halofantrine	28	2.38	1.88–3.00	30	1.69	1.36–2.11	<0.05
Pyrimethamine	14	41.8	6.2–281	20	81.4	14.5–456	NS
Dihydroartemisinin	33	1.25	0.99–1.57	32	0.979	0.816–1.18	NS

* Values are geometric mean 50% inhibitory concentrations (IC_{50}) in nanomoles/liter. n = number of isolates; *P* = probability of the difference of the mean IC_{50} of the chloroquine-sensitive and the chloroquine-resistant parasites (unpaired *t*-test); NS = not significant.

quine-sensitive isolates than against the chloroquine-resistant parasites. An opposite trend was observed with halofantrine, which was more active ($P < 0.05$) against the chloroquine-resistant isolates than against the chloroquine-sensitive isolates. Dihydroartemisinin was equally active ($P > 0.05$) against the chloroquine-sensitive and the chloroquine-resistant isolates.

The IC_{50} values for dihydroartemisinin were < 5 nM in all isolates tested. Two isolates had diminished *in vitro* sensitivity to quinine (IC_{50} = 624 and 633 nM), but none was quinine-resistant. One chloroquine-sensitive isolate showed elevated IC_{50} values for mefloquine (54 nM), halofantrine (10.9 nM), quinine (623 nM), pyrimethamine (854 nM), and dihydroartemisinin (4.56 nM). The other isolates had IC_{50} values below the cut-off values for mefloquine (< 20 nM) and halofantrine (< 6 nM). None of the isolates had an IC_{50} value > 60 nM for monodesethylamodiaquine. Pyrimethamine sensitivity was characterized by a wide range of IC_{50} values (0.24–8,940 nM).

A significant ($P < 0.05$) positive correlation was observed between the responses to dihydroartemisinin and mefloquine (r = 0.662), dihydroartemisinin and halofantrine (r = 0.284), chloroquine and monodesethylamodiaquine (r = 0.836), chloroquine and quinine (r = 0.405), and mefloquine and halofantrine (r = 0.423) (Table 2). A significant negative correlation ($P < 0.05$) was observed between the responses to chloroquine and halofantrine (r = −0.262). There was no significant correlation between the responses to dihydroartemisinin and chloroquine, monodesethylamodiaquine, quinine, or pyrimethamine.

DISCUSSION

At present, artemisinin derivatives do not have a well-defined role in antimalarial chemotherapy in Africa, with the possible exception for severe and complicated malaria.[5] Cameroonian Ministry of Public Health advocates the use of either chloroquine or amodiaquine for the first-line treatment of uncomplicated falciparum malaria, and sulfadoxine-pyrimethamine and quinine for the second-line and third-line treatment, respectively. Chloroquine resistance is highly prevalent in Yaoundé. In our previous studies, we have shown that the proportion of chloroquine-resistant *P. falciparum* infections is about 50%, *in vitro* and *in vivo*.[21,25,26] In contrast, amodiaquine is highly effective, *in vitro* and *in vivo*, against the clinical isolates obtained in Yaoundé.[27] Approximately 40% of the clinical isolates obtained in Yaoundé are resistant *in vitro* to pyrimethamine and have the mutant asparagine-108 allele in the dihydrofolate reductase gene, a genetic marker for pyrimethamine resistance.[21,28] Only a few isolates possess mutant alleles of the dihydropteroate synthase gene that are associated with sulfadoxine resistance.[29] Our preliminary studies have demonstrated the presence of *in vivo* resistance to sulfadoxine-pyrimethamine; the isolates obtained from these resistant cases carried multiple mutations in the dihydrofolate reductase and/or dihydropteroate synthase genes.[30] The rate of therapeutic failure with sulfadoxine-pyrimethamine is about 15% in Yaoundé (Ringwald P, unpublished data). There is no documented case of quinine resistance in Cameroon. Recent clinical studies have reported several recrudescent cases but these cases of therapeutic

TABLE 2

Correlation of *in vitro* responses of Cameroonian isolates of *Plasmodium falciparum* to dihydroartemisinin and standard antimalarial drugs*

Drug pair		Number of isolates	Correlation coefficient r	*P*
Dihydroartemisinin	Mefloquine	25	0.662	<0.05
Dihydroartemisinin	Halofantrine	58	0.284	<0.05
Dihydroartemisinin	Chloroquine	65	−0.058	NS
Dihydroartemisinin	Monodesethylamodiaquine	30	0.188	NS
Dihydroartemisinin	Quinine	60	0.176	NS
Chloroquine	Monodesethylamodiaquine	30	0.836	<0.05
Chloroquine	Quinine	60	0.405	<0.05
Chloroquine	Halofantrine	58	−0.262	<0.05
Mefloquine	Halofantrine	23	0.423	<0.05

* Spearman's rank-order correlation coefficient (*r*). NS = not significant.

failure were ascribed to underdosage.[31,32] Clinical trials in Yaoundé have also shown that pyronaridine, mefloquine, halofantrine, and artemether are highly effective (Ringwald P, unpublished data).[25,26,33]

In view of our data on the epidemiology of drug-resistant *P. falciparum,* artemisinin derivatives are currently not essential for malaria control in Yaoundé. We have nevertheless assessed the *in vitro* activity of dihydroartemisinin since artemether and artesunate are commercially available in Cameroon. Compared with other antimalarial drugs, dihydroartemisinin was almost as active as atovaquone against the African isolates (geometric mean IC_{50} = 0.89 nM [n = 35] and 0.91 nM [n = 26] against the chloroquine-sensitive and the chloroquine-resistant isolates, respectively).[34] These two drugs were the most potent test compounds among the currently available antimalarial drugs. Atovaquone is now being marketed in combination with proguanil as an antimalarial drug, but its usefulness in endemic countries may be limited by its high cost. Artemisinin derivatives are under various phases of clinical studies, and in Southeast Asia have become the first-line drugs for the treatment of multidrug-resistant *P. falciparum* infections.[3-5] Although artemisinin derivatives should be reserved for the treatment of multidrug-resistant *P. falciparum,* our data showed that dihydroartemisinin is equally active against the chloroquine-sensitive and the chloroquine-resistant isolates.

In our previous *in vitro* monitoring of artemether sensitivity of Cameroonian *P. falciparum* isolates in 1994–1995, the geometric mean IC_{50} value was 1.86 nM (95% confidence interval = 1.59–2.19 nM, range = 0.3–11.0 nM, n = 85).[21] The mean IC_{50} values for artemether were 2.37 nM (95% confidence interval = 1.90–2.94 nM, n = 32) and 1.61 nM (95% confidence interval = 1.30–2.00 nM, n = 53) in the chloroquine-sensitive and the chloroquine-resistant parasites, respectively. In another *in vitro* study conducted in 1997, we determined the baseline sensitivity data for artesunate in 61 clinical isolates.[22] The geometric mean IC_{50} for artesunate was 1.28 nM (95% confidence interval = 1.08–1.52 nM, range = 0.33–5.41 nM, n = 61). The mean artesunate IC_{50} values of the chloroquine-sensitive and the chloroquine-resistant isolates were 1.68 nM and 1.14 nM, respectively. In the present study using the isolates collected in 1997–1998, the geometric mean IC_{50} value for dihydroartemisinin was 1.11 nM. Shmuklarsky and others have obtained IC_{50} values for dihydroartemisinin ranging from 0.88 to 1.02 nM in three reference clones of *P. falciparum.*[35] These values are within the range of values found in our study. If we suppose that there was no shift in the sensitivity pattern to artemisinin derivatives in Yaoundé between 1994 and 1998 and compare the mean values obtained with different derivatives, dihydroartemisinin was 1.7 and 1.2 times more potent than artemether and artesunate, respectively.

Our comparison of different artemisinin derivatives is probably valid since a change in the sensitivity pattern to artemisinin derivatives within the past 5 years in Yaoundé is not likely for several reasons. First, artemisinin derivatives are rapidly eliminated. This pharmacokinetic feature does not favor the selection of mutant, resistant parasite populations in individual hosts.[36] Second, artemether and artesunate were introduced commercially in Cameroon in 1997. The volume of sales of these drugs, which are relatively costly for an average patient, remains limited, compared with the classical antimalarial drugs, which are perceived by most local populations to be still effective. Third, artemisinin derivatives are available only in official pharmacies. At the primary and secondary health care centres where a large majority of uncomplicated malarial infections are treated, these new drugs are not used. The prescribers of artemether and artesunate tend to be private and hospital practitioners. Fourth, natural resistance to artemisinin derivatives has not been reported in endemic countries before the introduction of the drugs. For these reasons, Cameroonian isolates have not yet been subjected to intense and haphazard drug pressure with artemisinin derivatives, as in Southeast Asia.

Several *in vitro* studies have shown that artemether and arteether are equipotent and that these two derivatives are about twice as active as the parent compound, artemisinin.[19,35,37,38] Artesunate is even more active *in vitro* than artemether and arteether; its activity is about four times greater than that of artemisinin.[19,38,39] Artesunate was almost as active as dihydroartemisinin in our studies. The IC_{50} values for artesunate and dihydroartemisinin were 1.66 nM and 1.79 nM in the W2/Indochina clone and 2.18 nM and 1.83 nM in the D6/Sierra Leone clone, respectively.[19] In the study of Wongsrichanalai and others, the geometric mean IC_{50} values of artesunate (3.22 nM) and dihydroartemisinin (3.24 nM) were also similar in 10 culture-adapted Vietnamese strains.[40] Artesunate is relatively unstable in aqueous solutions,[18] which may imply that the hydrolysis of artesunate to dihydroartemisinin during the 48-hr incubation at 37°C may explain why the mean IC_{50} values of these two derivatives are within a similar range. This hypothesis is consistent with the results of Bustos and others, which showed that of the four artemisinin derivatives (artesunate, artemether, arteether, and artemisinin) tested against Philippines isolates, artesunate (median IC_{50} = 0.9 nM) was the most potent *in vitro.*[38] These results raise the question regarding the choice of artemisinin derivatives for *in vitro* assays. The most reasonable choice seems to be the use of dihydroartemisinin for *in vitro* assays since this derivative is relatively stable and all currently used artemisinin derivatives and those that are under advanced phases of development are rapidly transformed into dihydroartemisinin in humans.[14-17]

The significant positive correlation between the *in vitro* responses to chloroquine and monodesethylamodiaquine or quinine and between the response to mefloquine and halofantrine is in agreement with *in vivo* resistance to amodiaquine, quinine, and halofantrine observed in Southeast Asia where a high level of resistance to chloroquine and mefloquine has emerged.[41,42] Cross-resistance between pyrimethamine and cycloguanil has also been observed *in vitro* and *in vivo.*[41,43] Cross-resistance patterns between these drugs may be, at least in part, explained on the basis of similar chemical structures. Our study suggests *in vitro* cross-resistance between dihydroartemisinin and aminoalcohols (mefloquine and halofantrine), which do not share any similar chemical features. A positive correlation was also obtained with other artemisinin derivatives and aminoalcohols in several *in vitro* studies.[37,38,44] The clinical and epidemiologic significance of the *in vitro* cross-resistance between these two classes of drugs is still unknown. The mechanism of action of artemisinin derivatives is thought to involve the interac-

tion between the drugs and hemin within the digestive vacuole of the parasite.[45–48] The iron-dependent decomposition of artemisinin generates free oxygen radicals that destroy the parasites. The 4-aminoquinolines and aminoalcohols also concentrate within the digestive vacuole but their precise mode of action has not been elucidated. Further studies are needed to understand the *in vitro* cross-resistance pattern between artemisinin and aminoalcohols.

In conclusion, the present study demonstrates the high *in vitro* activity of dihydroartemisinin against the Cameroonian clinical isolates. Dihydroartemisinin was more active *in vitro* than mefloquine and halofantrine. The responses of dihydroartemisinin and mefloquine or halofantrine were positively correlated, suggesting *in vitro* cross-resistance. A regular monitoring of the *in vitro* activity of dihydroartemisinin may be necessary in the future if local populations resort to artemisinin derivatives for antimalarial therapy.

Acknowledgments: We are grateful to Sister Solange Menard and her nursing and laboratory staff at the Catholic missionary Nlongkak Dispensary in Yaoundé for invaluable help.

Financial support: This investigation was supported by the French Ministry of Cooperation and Agence Universitaire de la Francophonie (AUF).

Authors' address: Pascal Ringwald, Jean Bickii, and Leonardo K. Basco, Institut de Recherche pour le Développement (IRD formerly ORSTOM) and Laboratoire de Recherches sur le Paludisme, Laboratoire Associé Francophone 302, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), BP 288, Yaoundé, Cameroon.

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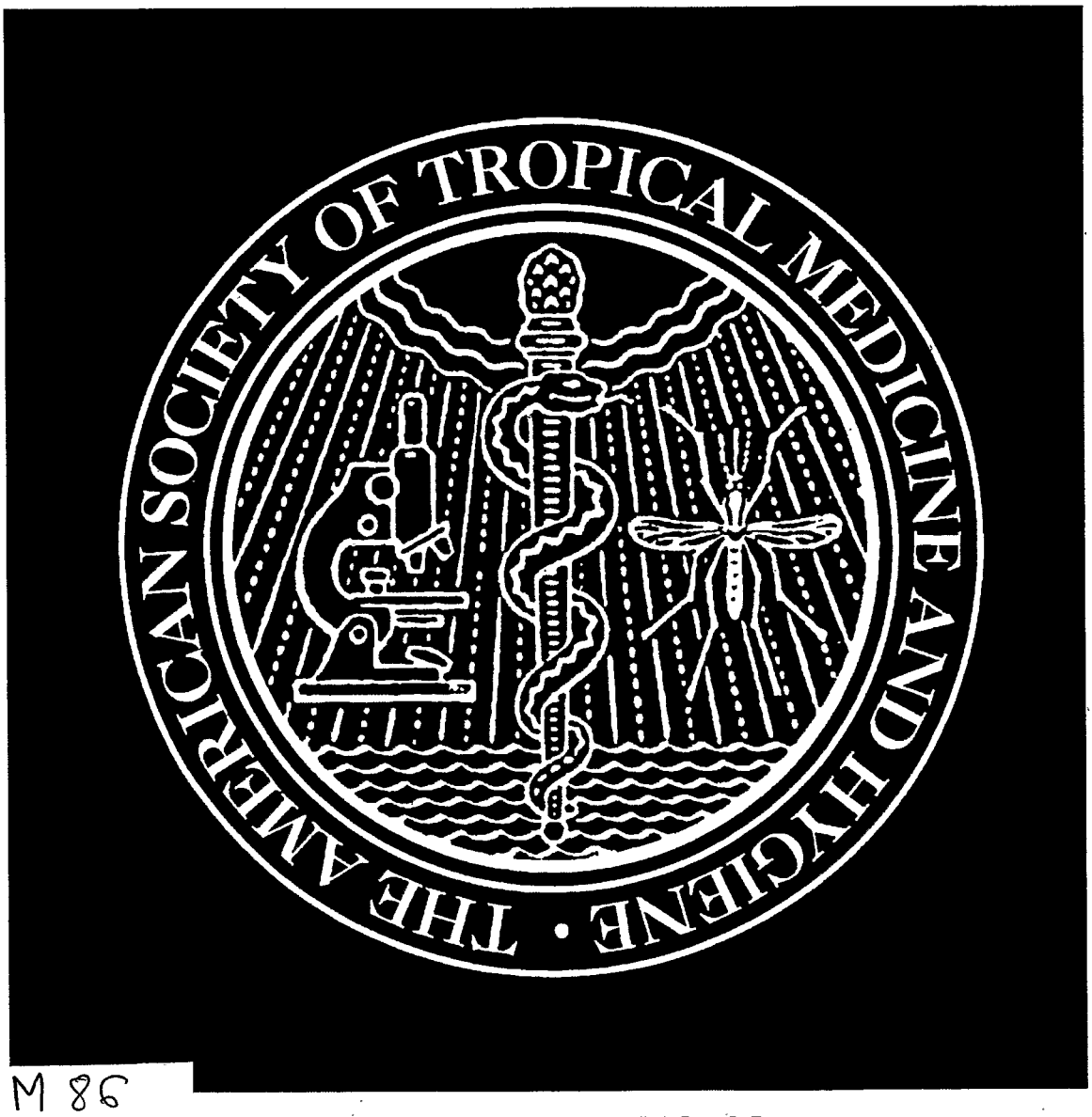

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AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

ISSN 0002-9637