

IN VIVO–IN VITRO MODEL FOR THE ASSESSMENT OF CLINICALLY RELEVANT ANTIMALARIAL CROSS-RESISTANCE

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Abstract. Cross-resistance may be considered one of the most important factors leading to decreased drug susceptibility of *Plasmodium falciparum*. The study aimed to determine whether clinically relevant cross-sensitivity of *P. falciparum* existed between artemisinin and mefloquine. Seventy-six patients with falciparum malaria were admitted and treated with artemisinin derivatives. Treatment response parameters were assessed and *in vitro* drug sensitivity tests were performed with artemisinin, mefloquine, quinine, and chloroquine. Distinct *in vitro* cross-sensitivity between artemisinin and mefloquine was observed ($\rho = 0.604$; $P < 0.001$). To assess the relevance of this finding for clinical cross-resistance, we used an analytical model based on the relation of *in vivo* treatment response parameters (fever, parasite and symptom clearance) to a single reference drug with *in vitro* drug sensitivity data of several other drugs. Artemisinin ($R = 0.554$; $P = 0.009$) and mefloquine ($R = 0.615$; $P = 0.002$) *in vitro* drug sensitivities were equally well reflected in the *in vivo* treatment response to artemisinin, thereby suggesting the clinical relevance of *in vitro* cross-sensitivity.

INTRODUCTION

Ever since the discovery of the first cases of chloroquine resistance along the Thai-Cambodian border in the late 1950s, Southeast Asia has played an important role as a focus for the occurrence of drug-resistant strains of *Plasmodium falciparum*.¹ The subsequent development of resistance to other antimalarials led to the introduction of mefloquine and finally its combination with artesunate as the standard regimen for the treatment of falciparum malaria in Thailand. In recent years, however, several studies describe a close *in vitro* activity correlation and cross-sensitivity between these 2 substances, which are currently presumably the 2 most important drugs in the treatment of multidrug-resistant falciparum malaria.^{2–4}

Cross-resistance may be considered one of the most important factors leading to decreased drug susceptibility of *P. falciparum*. In part because of this phenomenon, there has been need to periodically replace outdated treatment regimens with new antimalarials. The determination of antimalarial cross-resistance is generally based on the correlation of *in vitro* drug sensitivity parameters. *In vitro* correlations, however, do not necessarily reflect clinical cross-resistance. The aim of the study was therefore to find a new model for the determination of the clinical relevance of cross-resistance between antimalarial drugs on the basis of the relation of therapeutic response parameters to the *in vitro* drug sensitivity of *P. falciparum*. Because of the importance of these drugs, special emphasis was put on artemisinin derivatives and their relation to mefloquine.

MATERIALS AND METHODS

The study was designed to use *in vivo* and *in vitro* data acquired from patients included in controlled clinical trials at the Bangkok Hospital for Tropical Diseases (Faculty of Tropical Medicine, Mahidol University) obtained from June 1999 to March 2000. Written informed consent was obtained from all adult participants or from parents or legal guardians of minors. The study protocols were approved by the ethical

review board of the Faculty of Tropical Medicine, Mahidol University.

Seventy-six patients with a median age of 24.5 years of both sexes with microscopically confirmed *P. falciparum* infections were included in the study. All patients with plasmodial infections other than *P. falciparum* as well as women who were pregnant or lactating were excluded. Patients were excluded from the study if they had a history of antimalarial drug intake before admission: artemisinin within the past 7 days, 4-aminoquinolines within the past 14 days, pyrimethamine and sulfonamides within the past 28 days, or mefloquine within the past 56 days. History of drug intake before admission was taken, and Dill and Glazko's test for 4-aminoquinolines and the Lignin test for sulfonamides were performed to detect pretreatment.⁵

***In vivo* test procedure.** Patients were admitted to the Bangkok Hospital for Tropical Diseases and followed for 28 days to assess clinical findings, to eliminate the possibility of reinfection, and to observe cases of recrudescence. Patients were treated with a mean total dose of 11 mg/kg of artemisinin derivatives over a period of at least 3 days. Because of the large number of recrudescences to be expected with artemisinin monotherapy, artemisinin derivatives were combined with other antimalarials. To exclude a significant impact of the combination partners on the treatment response parameters, only drugs known to have a slow onset of action were used, and mefloquine was not administered before full parasite clearance. Body temperature, pulse, and respiration rates were recorded every 4 hr, and signs and symptoms were evaluated every day for the first 8 days.

Parasitological examination was performed by counting the parasites on thick and thin blood films every 12 hr. Parasite clearance time was defined as the time from the start of treatment until blood films were negative for asexual parasites of *P. falciparum* for the first time and remained negative for the next 48 hr. Fever clearance time was defined as the time from the start of treatment until the oral temperature dropped to below 37.5°C and remained below this temperature during the next 48 hr. A score of 0 to 4 (0 = none, 1 = mild, 2 = requires treatment, 3 = requires bed rest, and

TABLE 1
Correlation of *in vitro* activity at various effective concentrations for 4 antimalarial drugs*

Drug	n	ρ _{EC50}	P	ρ _{EC90}	P	ρ _{EC95}	P
ART-MEF	35	0.604	<0.001†	0.580	<0.001†	0.576	<0.001†
ART-QNN	35	0.206	>0.05	0.084	>0.05	0.063	>0.05
ART-CHL	35	0.211	>0.05	0.174	>0.05	0.241	>0.05
MEF-QNN	35	0.485	0.003†	0.348	0.04†	0.272	>0.05
MEF-CHL	35	0.212	>0.05	0.102	>0.05	0.062	>0.05
QNN-CHL	35	0.288	>0.05	0.282	>0.05	0.361	0.03†

* ART = artemisinin; CHL = chloroquine; EC = effective concentration (at 50, 90 and 95%); MEF = mefloquine; ρ = correlation coefficient; QNN = quinine.

† Statistically significant as determined by Spearman's rank correlation analysis.

4 = comatose) was assigned to the symptoms of all 76 patients, and the score was assessed every day within the first 8 days after the onset of treatment. An overall symptom score was calculated for every patient by adding daily scores, and the time was calculated until a complete clearance of symptoms was achieved (i.e., all scores were 0 and stayed at 0 for at least 48 hr).

In vitro test procedure. The *in vitro* tests for the measurement of the drug sensitivity of *P. falciparum* followed the standard methodology for the assessment of the inhibition of schizont maturation.^{6,7} Heparinized venous blood samples were drawn from every patient before the onset of treatment and mixed with RPMI 1640 medium in a dilution of 1 (5%) in 20. A total of 50 μL of the blood medium mixture was applied to each well of 96-well flat-bottomed microtiter plates pre-dosed with ascending quantities of artemisinin (0.15–150 pmol/well), mefloquine (2–128 pmol/well), quinine (4–256 pmol/well), and chloroquine (1–64 pmol/well). After an incubation of 24 hr at 37.5°C (± 0.5°C), the samples were harvested, and the slides were microscopically counted and later reexamined for the correctness of the readings. The artemisinin plates were prepared at the Department of Specific Prophylaxis and Tropical Medicine, Institute of Pathophysiology, University of Vienna, Austria; the mefloquine, quinine, and chloroquine plates were supplied by the World Health Organization, Regional Office for the Western Pacific, Manila, Philippines.

Statistical evaluation. Log-probit analysis of regression (log-dose and probit-response) was used to evaluate *in vitro* drug sensitivity tests.^{8,9} Correlations of clinical treatment response and *in vitro* response parameters were studied by means of standard correlation analysis and multiple linear regression models at a significance level of α = 5% (P < 0.05). Principal parameters used for multiple regression analysis were parasite clearance times (PCT), fever clearance times (FCT), and symptom clearance times (SCT) for *in vitro* drug response and effective concentrations (50, 90, and 95% effective concentrations [EC₅₀, EC₉₀, and EC₉₅]) for *in vitro* sensitivity. Nonparametric procedures were used for data that did not pass the Kolmogorov-Smirnov test for normal distribution.¹⁰

RESULTS

In vivo results. The median FCT for all 76 patients was 28.0 hr. Only 3 patients showed a FCT of > 100 hr, whereas 3 patients never developed fever (i.e., an oral temperature of ≥ 37.5°C). The median PCT₁₀₀ was 41.0 hr (range, 19.7–65.0 hr) and the mean clearance times for 50 and 90% of

the parasites (PCT₅₀ and PCT₉₀) were 8.6 hr (1.6–21.5 hr) and 15.3 hr (2.9–28.6 hr), respectively. The median SCT was found to be 72 hr within a range of 0–192 hr, and the median overall symptom score was 10 points (range, 0–165 points). No significant differences were found for any of these variables between the means of the 35 isolates, which were successfully tested for their *in vitro* drug susceptibility, as compared with the whole sample of 76 patients. The overall cure rate was 89%. Eight patients showed recrudescences within the observation period. The mean duration of time until the parasites reappeared in blood films was 17.6 ± 2.1 days (range, 15–21 days). All 8 patients showed parasite clearance within 120 hr and were therefore classified as RI cases with late recrudescence.

In vitro results. Parallel to the assessment of clinical improvement, 35 isolates were successfully tested for their *in vitro* susceptibility to artemisinin, mefloquine, quinine, and chloroquine. The geometric mean parasite density for these blood samples before treatment was 13,542 asexual parasites per microliter of blood. Individual ECs for all drugs were obtained by applying log-probit regression analysis to the culture results. The geometric mean of the individual EC₅₀, EC₉₀, and EC₉₅ values for artemisinin were 21.0, 78.2, and 112.4 nmol/L, respectively. The corresponding values for mefloquine were 704.5, 1,718.0, and 2,211.7 nmol/L; for quinine, 357.1, 862.2, and 1,106.9 nmol/L; and for chloroquine, 2,525.0, 6,878.4, and 9,826.3 nmol/L.

In vitro correlations. The same number of isolates (n = 35) was used to correlate the individual ECs of artemisinin, mefloquine, quinine, and chloroquine at the EC₅₀, EC₉₀, and EC₉₅ level, by means of Spearman's rank correlation analysis (Table 1). Highly significant correlations with coefficients (ρ) as high as 0.604 (P < 0.001) were found at all EC levels between the *in vitro* drug susceptibility to artemisinin and mefloquine, whereas no such relation was found with quinine (ρ_{EC50} = 0.206; P > 0.05) and chloroquine (ρ_{EC50} = 0.211; P > 0.05). Statistically significant activity correlations were also found between quinine and mefloquine at the lower EC levels (ρ_{EC50} = 0.485; P < 0.005) and between quinine and chloroquine at EC₉₅ (ρ_{EC95} = 0.361; P < 0.05).

In vivo-in vitro correlations. In the course of the correlation analysis between the *in vitro* results of artemisinin and the *in vivo* clinical response parameters, a highly significant association was found between effective concentrations and the fever clearance times at EC₅₀ (ρ_{EC50} = 0.551; P = 0.001), EC₉₀ (ρ_{EC90} = 0.557; P = 0.001), and EC₉₅ level (ρ_{EC95} = 0.575; P < 0.001). Artemisinin EC values did not significantly correlate with parasite and symptom clearance. Nevertheless, both parameters were included in the multivariate

TABLE 2

Multiple linear regression model for the assessment of clinically relevant cross-sensitivity based on the relation of *in vivo* (artemisinin [ART] treatment response) and *in vitro* (effective concentration [ECs]) data*

Drug	EC	n	df	R	Adjusted R ²	P
ART	EC ₅₀	35	3 + 31	0.554	0.240	0.009†
	EC ₉₀	35	3 + 31	0.559	0.246	0.008†
	EC ₉₅	35	3 + 31	0.578	0.269	0.005†
MEF	EC ₅₀	35	3 + 31	0.615	0.319	0.002†
	EC ₉₀	35	3 + 31	0.674	0.402	<0.001†
	EC ₉₅	35	3 + 31	0.668	0.392	<0.001†
QNN	EC ₅₀	35	3 + 31	0.237	0.035	>0.05
	EC ₉₀	35	3 + 31	0.238	0.034	>0.05
	EC ₉₅	35	3 + 31	0.259	0.023	>0.05
CHL	EC ₅₀	35	3 + 31	0.462	0.137	>0.05
	EC ₉₀	35	3 + 31	0.406	0.086	>0.05
	EC ₉₅	35	3 + 31	0.437	0.113	>0.05

* ART = artemisinin; CHL = chloroquine; df = degrees of freedom; EC = effective concentration (at 50, 90, and 95%); MEF = mefloquine; QNN = quinine; R = correlation coefficient. ART treatment response parameters (fever, parasite, and symptom clearance times) were used as independent variables and *in vitro* drug sensitivity data (effective concentrations of ART, MEF, QNN, and CHL were used as dependent variables).

† Statistically significant.

analysis to cover as many aspects of treatment response as possible. Correlation analysis was also performed between FCT, PCT, and SCT. No significant association was found between any of these parameters.

To assess the association of *in vitro* drug sensitivity with therapeutic response data, a multiple regression model was used with EC values of artemisinin as dependent variable and FCT, PCT, and SCT times as the independent variables (Table 2). A significant relationship was found for all effective concentrations of artemisinin with correlation coefficients of 0.554 ($P = 0.009$), 0.559 ($P = 0.008$), and 0.578 ($P = 0.005$) at EC₅₀, EC₉₀, and EC₉₅, respectively. This finding reflects the close relation between results from *in vitro* drug sensitivity tests with artemisinin and the corresponding clinical and parasitological treatment response. The regression analysis of mefloquine *in vitro* culture results with the artemisinin treatment response yielded even higher correlation coefficients of 0.615 ($P = 0.002$), 0.674 ($P < 0.001$), and 0.668 ($P < 0.001$). These results suggest that the *in vitro* correlation found between artemisinin and mefloquine is also reflected in artemisinin treatment response. For quinine and chloroquine, however, no significant correlations were found, which clearly reflects the lack of a correlation between *in vitro* results of artemisinin on the one hand and quinine and chloroquine on the other.

DISCUSSION

As compared with clinical trials, *in vitro* drug sensitivity tests offer comparatively objective results. *In vitro* drug sensitivity tests are not influenced by the patient's immune system or the formulation, absorption, or distribution of the drug, and they offer a simple way to assess cross-resistance between different antimalarials. However, relatively little is known about their clinical relevance. In this study, a combined *in vivo*-*in vitro* model was therefore used to assess the clinical relevance of *in vitro* cross-reactivity.

Because each of the individual treatment response param-

eters was found to be of limited value for the predictability of drug sensitivity, a more complex system was used, one based on a combination of FCT, PCT, and SCT. The aim was to find a quantitative measure to reflect *in vivo* drug sensitivity on a more comprehensive basis than individual clinical parameters. Effective concentrations were used as quantitative parameters of *in vitro* drug sensitivity. At the same time, the system should permit the correlation of *in vivo* and *in vitro* findings for a number of drugs other than the one tested *in vivo*. This rationale aimed to determine whether cross-sensitivities, which are found between *in vitro* results of 2 different drugs (such as between effective concentrations of artemisinin and mefloquine), are reflected in treatment response data and may therefore be of relevance for the development of clinical cross-resistance.

The naturally close correlation between artemisinin *in vivo* and *in vitro* results, which was found in the course of this analysis, reflects the dependence of treatment response on the drug sensitivity of *P. falciparum*. The similarly close relation of artemisinin *in vivo* findings with ECs of mefloquine, on the other hand, which were an almost perfect match for the close *in vitro* correlation of these substances, may only be explained by a distinct, clinically relevant cross-sensitivity. The fact that neither quinine nor chloroquine ECs showed any significant correlation with artemisinin treatment response indicates that in these cases, no clinical cross-resistance would have to be expected. The complex relation between the individual drugs and their relation to artemisinin treatment response is shown in Figure 1.

Some authors have suggested that the short half-life of artemisinin derivatives as well as their novel chemical structure would protect this class of antimalarials from the development of drug resistance. This study suggests, however, that future development of resistance of *P. falciparum* to artemisinin derivatives may not merely depend on the pharmacokinetic properties of artemisinin, but also on the future development of mefloquine resistance. The reason for the close relationship between artemisinin and mefloquine may possibly be found in parallels in their mode of action. This assumption is also supported by the fact that these drugs are synergistic when used in combination.¹¹⁻¹³ Furthermore, the role of the *pfmdr1* gene as a possible factor for the development of multidrug resistance, especially to mefloquine and artemisinin, has recently been proposed.^{14,15}

The findings from this study indicate a clinically relevant (*in vivo*) cross-sensitivity between artemisinin and mefloquine, similar to the one previously found *in vitro*. Such a relationship could have a significant impact on the future development of the artemisinin drug sensitivity of *P. falciparum*, especially if mefloquine is employed in highly malaria-endemic areas.

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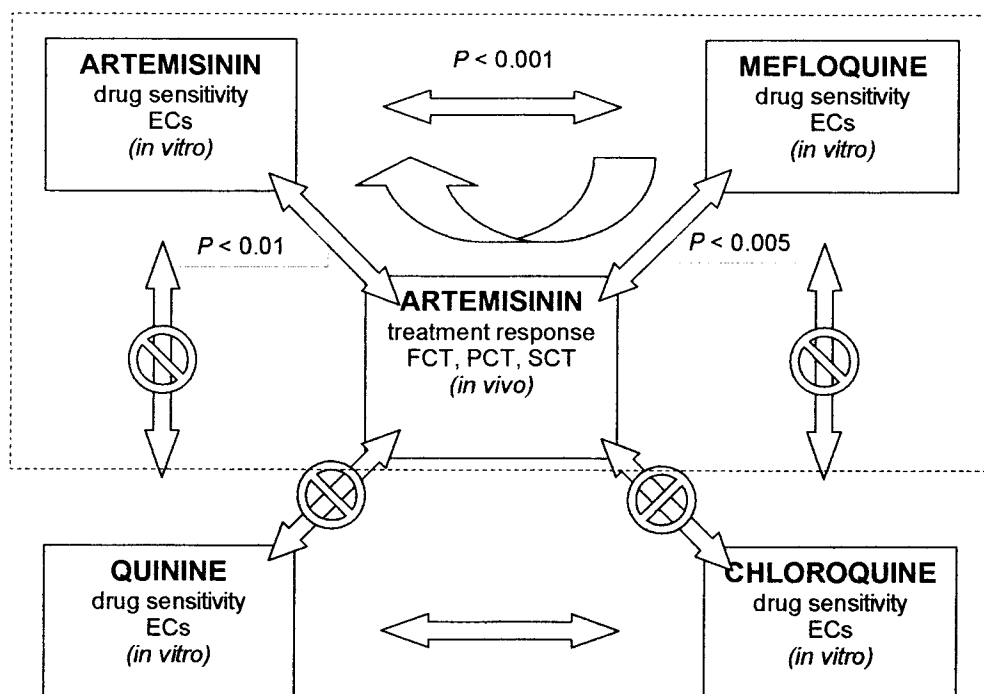


FIGURE 1. Model for the correlation of *in vitro* drug sensitivity of individual drugs with each other and with artemisinin treatment response parameters (arrows represent correlations, crossed-out arrows the lack of a correlation). The upper part displays the clinically relevant *in vivo* correlation between artemisinin and mefloquine. ECs = effective concentrations; FCT = fever clearance time; PCT = parasite clearance time; SCT = symptom clearance time.

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REFERENCES

1. Harinasuta T, Suntharasamai P, Viravan C, 1965. Chloroquine-resistant falciparum malaria in Thailand. *Lancet* 2 (7414): 657-660.
2. Basco LK, Le Bras J, 1993. In vitro activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* 49: 301-307.
3. Bustos MD, Gay F, Diquet B, 1994. In-vitro tests on Philippine isolates of *Plasmodium falciparum* against four standard antimalarials and four qinghaosu derivatives. *Bull World Health Organ* 72: 729-735.
4. Wongsrichanalai C, Wimonwattawatee T, Sookto P, Laobonchai A, Heppner DG, Kyle DE, Wernsdorfer WH, 1999. In vitro sensitivity of *Plasmodium falciparum* to artesunate in Thailand. *Bull World Health Organ* 77: 392-398.
5. WHO, 1979. *Instructions for Use of the WHO Test Kit for the Assessment of the Response of Plasmodium falciparum to Chloroquine*. WHO document MAP/79.1. Geneva, Switzerland: World Health Organization.
6. WHO, 1990. *In vitro Micro-test (Mark II) for the Assessment of the Response of Plasmodium falciparum to Chloroquine, Mefloquine, Quinine, Sulfadoxine/Pyrimethamine and Amodiaquine*. WHO document MAP/87.2, revision 1. Geneva, Switzerland: World Health Organization.
7. Wernsdorfer WH, 1980. Field evaluation of drug resistance in malaria. In vitro micro-test. *Acta Trop* 37: 222-227.
8. Litchfield JT, Wilcoxon F, 1949. A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 96: 99-113.
9. Wernsdorfer WH, Wernsdorfer MG, 1995. The evaluation of in vitro tests for the assessment of drug response in *Plasmodium falciparum*. *Mitt Oesterr Ges Tropenmed Parasitol* 17: 221-228.
10. Norman GR, Streiner DL, 1994. *Biostatistics: The Bare Essentials*. St. Louis: Mosby.
11. Na-Bangchang K, Tippawangkosol P, Thanavibul A, Ubalee R, Karbwang J, 1999. Pharmacokinetic and pharmacodynamic interactions of mefloquine and dihydroartemisinin. *Int J Clin Pharmacol Res* 19: 9-17.
12. Bwijo B, Alin MH, Abbas N, Wernsdorfer W, Bjorkman A, 1997. Efficacy of artemisinin and mefloquine combinations against *Plasmodium falciparum*. In vitro simulation of in vivo pharmacokinetics. *Trop Med Int Health* 2: 461-467.
13. Looareesuwan S, Wilairatana P, Viravan C, Vanijanonta S, Pitisutthithum P, Kyle DE, 1997. Open randomized trial of oral artemether alone and a sequential combination with mefloquine for acute uncomplicated falciparum malaria. *Am J Trop Med Hyg* 56: 613-617.
14. Duraisingh MT, Roper C, Walliker D, Warhurst DC, 2000. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the *pfmdr1* gene of *Plasmodium falciparum*. *Mol Microbiol* 36: 955-961.
15. Chaiyaroj SC, Buranakiti A, Angkasekwinai P, Looareesuwan S, Cowman AF, 1999. Analysis of mefloquine resistance and amplification of *pfmdr1* in multidrug-resistance of *Plasmodium falciparum* isolated from Thailand. *Am J Trop Med Hyg* 61: 780-783.