Published in final edited form as:
Trans R Soc Trop Med Hyg. 2000 ; 94(5): 537–544.

Plasmodium falciparum antimalarial drug susceptibility on the north-western border of Thailand during five years of extensive use of artesunate–mefloquine

A. Brockman[1,2], **R. N. Price**[1,3], **M. van Vugt**[1,2,5], **D. G. Heppner**[4], **D. Walsh**[4], **P. Sookto**[4], **T. Wimonwattrawatee**[4], **S. Looareesuwan**[2], **N. J. White**[2,3], and **F. Nosten**[1,2,3]

[1]Shoklo Malaria Research Unit, PO Box 46, Mae Sot 63110, Tak, Thailand [2]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand [3]Centre for Tropical Diseases, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford, UK [4]Department of Immunology and Medicine, Armed Forces Research Institute of Medical Science, Bangkok, Thailand [5]Division of Infectious Diseases, Tropical Medicine and AIDS, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Following a marked decline in the efficacy *in vivo* of mefloquine between 1990 and 1994, a combination of artesunate (4 mg/kg/d for 3 d) and mefloquine (25 mg/kg) has been used as first line treatment of uncomplicated falciparum malaria in camps for displaced persons located along the north-western border of Thailand. Antimalarial drug susceptibility of fresh isolates of *Plasmodium falciparum* from this population was evaluated using a radioisotope microdilution assay between 1995 and 1999. In total, 268 isolates were collected, of which 189 were from primary infections and 79 from recrudescent infections. The geometric mean 50% inhibitory concentration (IC_{50}) values from primary infections were: dihydroartemisinin 1·2 ng/mL, artesunate 1·6 ng/mL, artemether 4·8 ng/mL, atovaquone 0·4 ng/mL, lumefantrine 32 ng/mL, chloroquine 149 ng/mL, quinine 354 ng/mL, mefloquine 27 ng/mL and halofantrine 4·1 ng/mL. A significant positive correlation was found between the susceptibility *in vitro* to artesunate and quinine ($r = 0{\cdot}43$, $P < 0{\cdot}001$), mefloquine ($r = 0{\cdot}46$, $P < 0{\cdot}001$), and halofantrine ($r = 0{\cdot}51$, $P < 0{\cdot}001$). These levels of resistance *in vitro* are among the highest reported and confirm continuing high level multidrug resistance in this area. Despite intensive use of the combination between 1995 and 1999 there has been a significant improvement in mefloquine sensitivity ($P < 0{\cdot}001$) and artesunate sensitivity ($P < 0{\cdot}001$). This supports observations *in vivo* that the combination of artesunate and mefloquine has reversed the previous decline in mefloquine sensitivity.

Keywords

malaria; *Plasmodium falciparum*; drug resistance; dihydroartemisinin; artesunate; artemether; atovaquone; lumefantrine; chloroquine; quinine; mefloquine; halofantrine; Thailand

Address for correspondence: Dr François Nosten, Shoklo Malaria Research Unit, PO Box 46, Mae Sot 63110, Tak, Thailand; phone +66 55 531 531, fax +66 55 544 442, nosten@cm.ksc.co.th.

The views of the authors do not purport to reflect the position of the US Department of the Army or Department of Defense.

Europe PMC Funders Author Manuscripts

Introduction

Multidrug resistance in *Plasmodium falciparum* has emerged at an alarming rate in south-east Asia, and nowhere is the situation more serious than in Thailand. In this region, the level of resistance to chloroquine, amodiaquine and sulfadoxine–pyrimethamine precludes their use (Meek *et al.*, 1986). Susceptibility to quinine has also declined (Looareesuwan *et al.*, 1992; Pukrittayakamee *et al.*, 1994). In recent years, *P. falciparum* has developed resistance against the newer antimalarial compounds, including mefloquine (Nosten *et al.*, 1991) and halofantrine (ter Kuile *et al.*, 1993). By 1994, failure rates with high-dose mefloquine monotherapy (25 mg/kg) on the western border of Thailand were approaching 50%. Beginning in mid-1994, the standard regimen for the treatment of uncomplicated infections in camps for displaced persons on the western border has been a combination of high dose mefloquine (25 mg/kg) and 3 d of artesunate (12 mg/kg total dose) (MAS3) (Nosten *et al.*, 1994). This combination has consistently given cure rates approaching 100% (Price *et al.*; 1997; van Vugt *et al.*, 1998). The new combinations of artemether and lumefantrine (van Vugt *et al.*, 1998), and atovaquone–proguanil, also give excellent efficacy in this area. Since the introduction of combination therapy using one of the artemisinin derivatives, the incidence of *P. falciparum*, but not of *P. vivax*, has declined markedly in the camps, but not in the surrounding communities where such therapy is not widely used (Nosten *et al.*, 1999, in press). We have continuously monitored the antimalarial susceptibility *in vitro* of fresh isolates of *P. falciparum* obtained from patients with malaria in this population following the introduction of these antimalarial combinations containing an artemisinin derivative.

Materials and Methods

Isolates of *P. falciparum*

Parasite isolates were obtained between March 1995 and July 1999 from patients with acute falciparum malaria attending the clinics of the Shoklo Malaria Research Unit (SMRU). Patients were recruited from 2 camps (Shoklo and Maela) for displaced persons of the Karen ethnic minority situated in an area of forested hills on the north-western border of Thailand. The epidemiology of malaria in this area has been described elsewhere (Luxemburger *et al.*, 1996). Isolates were collected regardless of presenting parasitaemia, provided that the patients agreed to give a blood sample and they, or their attendant parent or guardian, gave informed consent. Isolates were categorized into primary or recrudescent infections depending on the malaria history of the patient. Patients presenting with an infection of falciparum malaria within 42 d of MAS3 treatment were considered to have a recrudescent, i.e. resistant, infection, though the possibility of its being a new infection could not be discounted. Genetic studies in this population indicated that 66% of infections from non-pregnant patients that recurred within 42 d were true recrudescences (A. Brockman, unpublished observations).

The isolates were transported within 4-8 h at room temperature to the laboratory in Mae Sot in Thailand, 1–2 h drive from the study sites, and then either tested immediately or set up in continuous culture.

Drugs

Chloroquine diphosphate, quinine citrate, mefloquine hydrochloride, halofantrine hydrochloride, artesunate, and dihydroartemisinin were obtained from the Walter Reed Army Institute of Research (WRAIR), Washington DC, USA, courtesy of Dr D. E. Kyle. Artemether and lumefantrine were obtained from Novartis Pharma Inc., Basel, Switzerland. Atovaquone (566C80) was obtained from Glaxo–Wellcome, UK.

Chloroquine, quinine, mefloquine, halofantrine, artesunate and dihydroartemisinin were dissolved in 75% ethanol to form stock solutions. Artemether and lumefantrine were dissolved in a 1:1:1 (w/v) mixture of ethanol, Triton-X (Sigma) and linoleic acid (Sigma) (W. Wernsdorfer, personal communication) and sterilized by ultrafiltration before being diluted in culture media. Atovaquone, with its low aqueous solubility, was dissolved in absolute ethanol by sonication. All drugs were dissolved initially at a concentration of 1 mg/mL. The solvent in the final concentrations had no effect on parasite growth when compared to culture media without the addition of drug (data not shown).

Drug sensitivity testing

A microdilution radioisotope method was used to assess *P. falciparum* antimalarial drug susceptibility (Webster *et al.*, 1985). Briefly, 5 mL of blood were taken by venepuncture into sterile tubes containing 0·05 mL of 15% ethylenediaminetetraacetic acid (EDTA; Becton Dickinson). Plasma and buffy coat were removed after centrifugation and the red blood cells were washed 3 times in phosphate-buffered saline (PBS). Isolates were set up in preculture or cultured directly in the presence of antimalarial drugs, depending on presenting parasitaemia. If at least 90% of the parasites were synchronous at the ring stage and the parasitaemia was greater than or equal to 0·5% infected red blood cells (IRBC), the isolate was considered ready for the drug assay. If these criteria were not met, infected erythrocytes were cultured in complete RPMI-1640 medium (Gibco BRL) with 10% heterologous serum of the patient's blood group until the target parasitaemia of 0·5% was reached. The culture medium was changed daily and incubation was at 37·5°C in the presence of 5% CO_2, 5% O_2 and 90% N_2.

Drug testing *in vitro* using the radioisotope microdilution technique

The cultured or fresh isolates were adjusted to an optimum density of 0·5–1·0% IRBC and a haematocrit of 1·5% using fresh washed group O erythrocytes and complete RPMI-1640 medium with 10% heat-inactivated AB sera (Sigma, lot no. 45H4608). The suspension of infected erythrocytes was dispensed (200 μL) into the wells of a standard microtitre plate (96 flat-bottom wells, 8 × 12 matrix) containing duplicate serial dilutions of the antimalarial drugs. Each isolate was tested once in duplicate for all drugs. A two-fold serial dilution for a total of 7 concentrations over a 64-fold range was made for each drug. The pre-dosed plates were made up in bulk at regular intervals and frozen at −25°C until used. The volume of drug solution added to each well was 25 μL.

Following incubation for 24 h, the microtitre plates were pulsed with [^{3}H]hypoxanthine (specific activity 7·3 Ci/mmol) (Amersham Life Sciences) by addition of 25 (μL (0·625 (μCi) of the isotope solution to each well. After a further 18 h incubation, the plates were

frozen and transported on dry ice to Bangkok, for harvesting on an automated cell harvester (Tomtec, Connecticut, USA). The glass fibre papers were dried, placed in sample bags, and 5 mL of a toluene-based scintillation fluid added. Radioactivity was determined in a liquid scintillation spectrometer (Wallac, Finland). An accessory program was used to compute quench-corrected absolute disintegrations per minute (DPM) of the tritium-labelled samples.

The reproducibility of 50% inhibitory concentration (IC_{50}) measurements for 6 antimalarial drugs was assessed in 1996 and again in 1998 using the cloned K1 isolate of *P. falciparum*. Inter-laboratory variability was assessed by comparing responses *in vitro* of 6 cryopreserved field isolates in both the SMRU laboratory and the AFRIMS laboratory in Bangkok. Comparison of pre-treated with frozen drug plates was done by assaying 4 field isolates on both types of plates.

Data analysis

Concentration–response data were analysed by a nonlinear regression function to determine the IC_{50}, defined as the concentration of the drug which inhibited 50% of the uptake of [^{3}H]hypoxanthine into the nucleoprotein of the parasites in drug-free control wells. The program WinNonlin™ (Scientific Consulting, Inc., Durham, North Carolina, USA) was used to calculate the parameters of the concentration–response curve using an inhibitory effect sigmoid E-max model.

IC_{50} results were excluded if there was less than a 5-fold increase in DPM between the drug-free control wells containing infected and uninfected red blood cells. All other modelled curves were assessed by 2 independent observers. All drug concentrations are expressed as nano-grams/millilitre (ng/mL). To convert to nanomolar concentrations (nmol/L), the factors are: chloroquine 515·9, quinine 516·5, mefloquine 414·8, halofantrine 536·9, artesunate 384·4, artemether 298·0, dihydroartemisinin 284·4, lumefantrine 529·0 and atovaquone 366·8.

Statistical analysis

Data were analysed using the program SPSS for Macintosh (SPSS Inc., Chicago, Illinois, USA). Proportions were compared using the χ^2 test with Yates's correction or Fisher's exact test. Baseline parasitaemia and IC_{50} values were log-transformed and comparisons made using Student's *t* test or one-way analysis of variance. Reproducibility *in vitro* (comparing the K1 isolate assayed in 1996 with 1998) was assessed using the Mann–Whitney *U* test. Inter-laboratory variability (SMRU and AFRIMS) and drug plate comparisons were assessed by the Wilcoxon signed rank test. Cross-resistance *in vitro*, temporal trends and molar equivalents of the artemisinin derivatives were analysed on log-transformed data by the Pearson correlation coefficient (*r*). The independence of cross correlations between drugs after controlling for the other antimalarial compounds was assessed by multiple linear regression. For temporal trends, time was defined by the month of collection and assessed after stratifying by primary and recrudescent infections. Statistical significance was assumed if $P < 0{\cdot}05$. For multiple comparisons, the level of significance was adjusted using the Bonferroni correction.

Results

Between February 1995 and August 1999, 268 fresh isolates of *P. falciparum* were collected and assayed *in vitro* for drug susceptibilities. Clinical and laboratory variables are shown in Table 1. More details of the changes in malaria epidemiology and therapeutic responses in this area between 1986 and 1999 are given by Nosten *et al.* (in press).

Quality control

The number of IC_{50} values that were rejected because of inadequate assays *in vitro* for the drugs tested were as follows: 3·1% (6/189) for chloroquine, 3·2% (8/247) for quinine, 4·4% (11/250) for mefloquine, 6·8% (13/192) for halofantrine, 3·8% (10/260) for artesunate, 6·6% (9/137) for dihydroartemisinin, 4% (2/50) for artemether, 13% (6/46) for atovaquone, and 3·0% (1/33) for lumefantrine. There was no significant difference between the IC_{50} values of the K1 cloned isolate determined in 1996 and those in 1998 (Table 2). The geometric mean values of the pooled IC_{50} results were as follows: chloroquine 243·1 (SD = 64·9) ng/mL, quinine 614·8 (SD = L79·0) ng/mL, mefloquine 24·5 (SD = 3·8) ng/mL, artesunate 5·1 (SD = 0·85) ng/mL, dihydroartemisinin 3·2 (SD = 0·50) ng/mL, artemether 3·9 (SD = 1·2) ng/mL. No significant difference in the responses *in vitro* was observed with any of the drugs assessed when comparing isolates tested on freshly made drug plates or pre-frozen plates ($P > 0·3$). There was also no significant difference in the IC_{50} values of any drugs tested on cryopreserved isolates whether processed at SMRU or AFRIMS ($P > 0·3$).

Confounding factors

In total, 32 (12%) isolates were derived from Shoklo camp and 236 from Maela camp. IC_{50} values were significantly higher for isolates from Shoklo for both mefloquine (geometric mean = 48·2 ng/ml [95% confidence intervals (95% CI) 36·4–63·8] vs. 29·0 ng/ml [95% CI 25·7–32·7]; $P = 0·04$) and halofantrine (13·0 ng/mL [95% CI 9·0–18–8] vs. 4·4 ng/mL [95% CI 3·7–5·3]; $P = 0·001$), but not for the other drugs tested. All the samples from Shoklo were collected before 1997, whereas 73% (172/236) of the samples from Maela were collected after this. After correcting for the year of the collection, there was no difference in response *in vitro* between the 2 study sites. Data were available from the camps concerning the movements of 145 of the patients in the 2 months before their malaria infection. Thirty-five (24%) patients were residents who had never left the camps, 88 (61%) were workers who spent some of that time engaged in work activities outside of the camp, and 22 (15%) were visitors who did not normally live in the camps. There was no difference in response *in vitro* to any of the drugs of isolates obtained from these different groups of patients.

Of the isolates examined, 189 (70%) came from patients with primary infections and 79 (30%) from recrudescent infections. Of the latter, 43 (54%) had received prior treatment with regimens containing mefloquine plus artesunate, 18 (23%) with lumefantrine plus artemether, 6 (8%) with artesunate monotherapy, 4 with artesunate plus doxycycline, 5 with atovaquone, 2 with mefloquine and 1 with quinine. Parasite isolates from recrudescent infections had significantly higher IC_{50} values, compared to primary infections, for mefloquine ($P = 0·008$), halofantrine ($P = 0·008$), artesunate ($P = 0·016$) and atovaquone ($P = 0·04$); see Table 3.

Overall, 68 isolates (25%) underwent short-term culture *in vitro* for a median of 4 d (range 2–13) to achieve the required minimum parasitaemia (0·5%) before drug sensitivity testing. Culturing was required more often with recrudescent than with primary isolates [56% (44/79) vs. 13% (24/189); $P < 0·001$] and reflected the lower presenting parasitaemias of the recrudescent isolates (geometric means 11 190 vs. 56 325 parasites per mm^3; $P< 0·001$). Isolates requiring prior culturing had significantly higher IC_{50} values than the isolates assayed immediately with mefloquine ($P = 0·008$), halofantrine ($P< 0·001$) and artesunate ($P = 0·008$). This difference was no longer apparent after stratifying by primary versus recrudescent infections.

Susceptibility *in vitro* to the artemisinin derivatives

The geometric mean molar IC_{50} values of artemether were significantly higher than those of both artesunate (16·9 nmol/L vs. 5·0 nmol/L) and dihydroartemisinin(DHA) (14·8 nmol/L vs. 6·3 nmol/L) when assayed against the same isolates (geometric mean ratios 3·4 [95% CI 2·6–4·3] and 2·3 [95% CI 1·8–3·1] respectively; $P < 0·001$). The discrepancy between artemether and artesunate was correlated significantly with the IC_{50} of artemether ($r_s = 0·93$; $P < 0·001$). Although there was no significant difference between the molar IC_{50} values of artesunate and DHA in a paired analysis, the difference between the IC_{50} values of the 2 drugs was correlated significantly with that of artesunate ($r_s = 0·80$; $P< 0·001$). When comparing the variance of the assay results of the K1 isolate, the coefficient of variation (CV) of the IC_{50} values was greatest for artemether (30·3%) and least for DHA (15·2%) ($F = 5·76$, $P = 0·01$). There was no significant difference between the CV for artesunate (16·4%) and either artemether or DHA.

Cross resistance *in vitro*

There were significant positive correlations between the IC_{50} values for mefloquine and the following drugs: quinine ($r = 0·62$; $P< 0·001$), halofantrine ($r = 0·65$; $P < 0·001$), artesunate ($r = 0·46$; $P < 0·001$) and DHA ($r = 0·31$; $P = 0·02$); see Table 4 and Fig. 1. In a multivariate model, however, mefloquine was correlated significantly with only quinine and halofantrine ($P< 0·001$), but not artesunate. The IC_{50} value for artesunate was independently correlated with that for halofantrine ($P < 0·001$) and quinine ($P = 0·003$). There was a positive correlation between the sensitivities of the following pairs of artemisinin derivatives: artemether and artesunate ($r = 0·51$; $P = 0·01$), dihydroartemisinin and artesunate ($r = 0·40$; $P < 0·001$), and dihydroartemisinin and artemether ($r = 0·70$; $P = 0·04$). The IC_{50} values of chloroquine, atovaquone and lumefantrine were not correlated significantly with each other or with any of the other antimalarial drugs tested.

Temporal trends

With the exception of chloroquine, there has been no decline in susceptibilities *in vitro* over time for any of the drugs (Figs 1 and 2). In fact, there was a significant trend for increased susceptibility over time (lower IC_{50} values) for mefloquine ($r=−0·26$; $P = 0·001$), halofantrine ($r = −0·49$; $P < 0·001$) and artesunate ($r = −0·32$; $P < 0·001$). These trends remained significant after controlling for primary versus recrudescent infections. In contrast,

sensitivities *in vitro* to chloroquine declined between 1997 and 1999 for both primary and recrudescent infections (r = 0·66 and 0·67, respectively; $P < 0·001$).

Discussion

The results obtained *in vitro* in this large study confirmed that isolates of *P. falciparum* from this region are among the most multidrug-resistant in the world. When comparing mean IC_{50} values assessed by similar methods, drug resistance profiles to chloroquine, halofantrine, artesunate and artemether are consistently higher in this area than elsewhere in south-east Asia and Africa (Wongsrichanalai *et al.*, 1992a, 1992b, 1997; Basco & Le Bras, 1994; Ringwald *et al.*, 1996; Gay *et al.*, 1997; Heppner & Ballou, 1998). Only quinine had higher reported IC_{50} values, in Cambodia (Basco & Le Bras, 1994).

Significant correlations were observed between DHA and artesunate (r = 0·40; $P < 0·001$) and DHA and artemether (r = 0·70; P = 0·04). These were to be expected, since artesunate undergoes rapid de-esterification and artemether demethylation *in vivo* to form DHA, the active metabolite, and all the drugs share a common mode of action. In molar terms, artemether IC_{50} values were more than twice as high as the corresponding values for both artesunate and DHA and they were more variable. Furthermore, the discrepancy between the artemether assay and those of the other 2 derivatives was increased significantly compared with the absolute IC_{50} of artemether (r_s = 0·93 for artesunate and r_s = 0·82 for DHA; $P <$ 0·001). This may reflect methodological difficulties in using artemether, a lipophilic hydrophobic compound, in drug assays *in vitro*. Reports of reduced susceptibility *in vitro* to these peroxidic antimalarial drugs, particularly when artemisinin or artemether are the only drugs evaluated, may be explained by problems with dissolution of these drugs, rather than true resistance. The variance for DHA susceptibility estimates was significantly lower than that for artemether (P = 0·01) and also for artesunate, although this latter did not reach statistical significance (P = 0·1). As artesunate is intrinsically unstable at neutral pH and dissociates to DHA *in vitro*, it seems marginally preferable to use DHA for assessments *in vitro*.

Cross-correlation of IC_{50} values between the quinoline drugs (mefloquine, quinine and halofantrine) and the artemisinin derivatives, a structurally distinct class of drugs, has been noted previously (Basco *et al.*, 1993; Wongsrichanalai *et al.*, 1999; Ringwald *et al.*, 1996) and may indicate a common mechanism for acquiring resistance (Price *et al.*, 1999). Although cross resistance *in vitro* does not necessarily predict cross resistance *in vivo*, since individual immune status and pharmacokinetic factors are not taken into account, a high level of cross resistance *in vitro* may have clinical significance in an endemic region where resistance to an antimalarial drug is firmly established. This has led some to suggest that the sensitivity of *P. falciparum* to artemisinin derivatives may be compromised by mounting resistance to mefloquine (Wongsrichanalai *et al.*, 1999). This would be more likely to operate through loss of protection by mefloquine in combination regimens, leaving the artemisinin derivative ‘unprotected’ from selection pressures, and would require a very high level of mefloquine resistance. The evidence from this area of the western border of Thailand, where combinations have been used systematically, demonstrates the reverse: continued high efficacy of the artemisinin derivatives and the combination of mefloquine

Europe PMC Funders Author Manuscripts

and artesunate *in vivo*, and declining resistance to mefloquine and the artemisinin derivatives *in vitro*. The low level cross resistance *in vitro* between mefloquine and artesunate is more than counterbalanced by the mutual protection from resistance selection in combination therapy. Nevertheless, in adjacent areas where combination treatment is not used systematically (Nosten *et al.*, in press), resistance to mefloquine may progress further and could eventually compromise the combination. Resistance to chloroquine followed the opposite pattern to mefloquine—a well known inverse relationship. This is unlikely to have resulted from altered chloroquine use; the incidence of *P. vivax* malaria, now the predominant species in this area, has changed much less than that of falciparum malaria.

Parasite isolates defined as recrudescent, or drug treatment failures, had lower presenting parasitaemias than isolates from primary infections, largely because of active case detection. Parasite isolates from treatment failures, predominantly following mefloquine or lumefantrine in combination with an artemisinin derivative, had significantly higher IC_{50} values for mefloquine, halofantrine and atovaquone, and this was still apparent for mefloquine and halofantrine after correcting for time trend differences (Table 3 and Figs 2 and 3). Although a similar trend was seen for the other drugs tested (with the exception of chloroquine), this did not reach statistical significance. The unavoidable misclassification of new infections as recrudescences will underestimate the difference in drug sensitivities between primary and recrudescent isolates and this would decrease the study's power of detecting a true difference. Nevertheless, this points to the powerful selective pressure exerted by antimalarial drugs towards the emergence of resistance through both preferential survival, and thus transmission of resistant parasites, and the much rarer selection of *de novo* mutants with reduced susceptibility (White, 1999).

During the 5 years period of this study, there has been no decline in the susceptibility *in vitro* to mefloquine (Figs 2 and 3); in fact, the sensitivity to this drug has increased significantly. This contrasts with a 40% decrease in efficacy *in vivo* to mefloquine in the 5 years before the study (Price *et al.*, 1997). Cure rates with artesunate–mefloquine remain almost 100%. The protective effect of artesunate on mefloquine in the combination regimen derives from the consistent efficacy of artesunate against resistant parasites, ensuring high cure rates, the considerable reduction of parasite biomass reducing the chance of selecting mefloquine-resistant mutant parasites, and reduction in transmissibility (Price *et al.*, 1996; White, 1998). These factors reduce the selection and differential transmission advantage of mefloquine-resistant parasites (White, 1999). In this study site the apparent reversal of resistance probably resulted from eradication of the mefloquine-resistant isolates and repopulation with more sensitive 'wild type' parasites from surrounding areas (Nosten *et al.*, in press). Parasites from south-east Asia may be inherently more likely to develop drug resistance than those from other endemic areas (Rathod *et al.*, 1997) and this may, in part, account for the speed with which multidrug-resistant malaria has emerged in this region. There is concern that resistance to artemisinin may develop with the increasing use of these drugs. Although there has been no published report of resistance *in vitro* to any of the artemisinin derivatives, resistant strains of *P. falciparum* have been established (though not sustained) *in vitro* (Inselburg, 1985). Our monitoring has failed to reveal evidence of a

decline in the sensitivity of artesunate despite extensive use over the 5 years study period. In fact, sensitivity has increased.

The artemisinin derivatives remain an integral part of the treatment of multidrug-resistant falciparum malaria. Although there is no evidence that resistance to the artemisinin derivatives exists, caution is warranted and continued monitoring, both *in vivo* and *in vitro*, is essential.

Acknowledgements

We are very grateful to the Karen staff of the Shoklo Malaria Research and to the Department of Immunology and Medicine, AFRIMS, for their kind assistance and support. We also thank Julie Simpson for statistical advice. D. Gray Heppner is Chief, Department of Immunology at the Walter Reed Army Institute of Research, Washington, DC, USA. This study was part of the Wellcome–Mahidol University Oxford Tropical Medicine Research Programme, supported by the Wellcome Trust of Great Britain.

References

Basco LK, Le Bras J. *In vitro* activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. American Journal of Tropical Medicine and Hygiene. 1993; 49:301–307. [PubMed: 8372953]

Basco LK, Le Bras J. *In vitro* susceptibility of Cambodian isolates of *Plasmodium falciparum* to halofantrine, pyronaridine and artemisinin derivatives. Annals of Tropical Medicine and Parasitology. 1994; 88:137, 144. [PubMed: 8067809]

Gay F, Ciceron L, Litaudon M, Bustos MD, Astagneau P, Diquet B, Danis M, Gentilini M. *In vitro* resistance of *Plasmodium falciparum* to qinghaosu derivatives in West Africa. Lancet. 1997; 343:850–851. [PubMed: 7908091]

Heppner DG, Ballou WR. Malaria in 1998: recent advances in diagnosis, drugs and vaccine development. Current Opinion in Infectious Diseases. 1998; 11:519–530. [PubMed: 17033417]

Inselburg J. Induction and isolation of artemisinine-resistant mutants of *Plasmodium falciparum*. American Journal of Tropical Medicine and Hygiene. 1985; 34:417–418. [PubMed: 3890571]

Looareesuwan S, Wilairatana P, Vanijanonta S, Kyle D, Webster K. Efficacy of quinine-tetracycline for acute uncomplicated falciparum malaria in Thailand. Lancet. 1992; 339:369. [PubMed: 1346445]

Luxemburger C, Thwai KL, White NJ, Webster HK, Kyle DE, Maelankirri L, Chongsuphajaisiddhi T, Nosten F. The epidemiology of malaria in a Karen population on the western border of Thailand. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1996; 90:105–111. [PubMed: 8761562]

Meek SR, Doberstyn EB, Gauzer BA, Thanapanich C, Nordlander E, Phuphasan S. Treatment of falciparum malaria with quinine and tetracycline or combined mefloquine/sulfadoxine/pyrimethamine on the Thai–Kampuchean border. American Journal of Tropical Medicine and Hygiene. 1986; 35:246–250. [PubMed: 3513642]

Nosten F, ter Kuile F, Chongsuphajaisiddhi T, Luxemburger C, Webtser HK, Edtsein M, Phaipun L, Thew KL, White NJ. Mefloquine-resistant falciparum malaria on the Thai-Burmese border. Lancet. 1991; 337:1140–1143. [PubMed: 1674024]

Nosten F, Luxemburger C, ter Kuile F, Woodrow C, Eh JP, Chongsuphajaisiddhi T, White NJ. Treatment of multi-dnig resistant *Plasmodium falciparum* malaria with 3-day artesunate–mefloquine combination. Journal of Infectious Disease. 1994; 170:971–977.

Nosten F, Hien TT, White NJ. Use of artemisinin derivatives for the control of malaria. Médecine Tropicale. 1999; 58:45–49. [PubMed: 10212897]

Nosten F, van Vugt M, Price R, Luxemburger C, Brockman A, McGready R, ter Kuile FO, Looareesuwan S, White NJ. Effects of artesunate–mefloquine combination on the incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. Lancet. in press.

Europe PMC Funders Author Manuscripts

Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, White NJ. Effects of artemisinin derivatives on malaria transmissibility. Lancet. 1996; 347:1654, 1658. [PubMed: 8642959]

Price RN, Nosten F, Luxemburger C, van Vugt M, Phaipun L, Chongsuphajaisiddhi T, White NJ. Artesunate/mefloquine treatment of multi-drug resistant falciparum malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1997; 91:574–577. [PubMed: 9463672]

Price RN, Cassar C, Brockman A, van Vugt M, Duraisingh M, Nosten F, Krishna S. The *pfmdr 1* gene is associated with a multidrug resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrobial Agents and Chemotherapy. 1999; 43:2943–2949. [PubMed: 10582887]

Pukrittayakamee S, Supanaranond W, Looareesuwan S, Vanijananta S, White NJ. Quinine in severe falciparum malaria: evidence of declining efficacy in Thailand. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1994; 88:324–327. [PubMed: 7974679]

Rathod PK, McErlean T, Pei-Chieh L. Variations in frequencies of drug resistance in *Plasmodium falciparum*. Proceedings of the National Academy of Sciences of the USA. 1997; 94:9389–9393. [PubMed: 9256492]

Ringwald P, Bickii J, Basco LK. In vitro activity of antimalarials against clinical isolates of *Plasmodium falciparum* in Yaoundé, Cameroon. American Journal of Tropical Medicine and Hygiene. 1996; 55:254–258. [PubMed: 8842110]

Ter Kuile F, Dolan G, Nosten F, Edstein MD, Luxemburger C, Phaiphun L, Chongsuphajaisiddhi T, Webster HK, White NJ. Halofantrine versus mefloquine in treatment of multidrug-resistant falciparum malaria. Lancet. 1993; 341:1044–1049. [PubMed: 8096956]

Van Vugt M, Brockman A, Gemperli B, Luxemburger C, Gathmann I, Royce C, Slight T, Looareesuwan S, White NJ, Nosten F. Randomized comparison of artemether–benflumetol and artesunate–mefloquine in treatment of multidrug-resistant falciparum malaria. Antimicrobial Agents and Chemotherapy. 1998; 42:135–139. [PubMed: 9449273]

Webster HK, Boudreau EF, Pavanand K, Yongvanitchit K, Pang LW. Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Thailand using a micro-dilution radioisotope method. American Journal of Tropical Medicine and Hygiene. 1985; 34:228–235. [PubMed: 3885770]

White NJ. Preventing antimalarial drug resistance through combinations. Drug Resistance Updates. 1998; 1:3–9. [PubMed: 17092790]

White NJ. Antimalarial drug resistance and combination chemotherapy. Philosophical Transactions of the Royal Society of London, series B. 1999; 354:739–749. [PubMed: 10365399]

Wongsrichanalai C, Webster HK, Wimonwattrawatee T, Sookto P, Chuanak N, Timasarn K, Wernsdorfer WH. *In vitro* sensitivity of *Plasmodium falciparum* isolates in Thailand to quinine and chloroquine, 1984–1990. Southeast Asian Journal of Tropical Medicine and Public Health. 1992a; 23:533–536. [PubMed: 1488713]

Wongsrichanalai C, Webster HK, Wimonwattrawatee T, Sookto P, Chuanak N, Timasarn K, Wernsdorfer WH. Emergence of multidrug–resistant *Plasmodium falciparum* in Thailand: *in vitro* tracking. American Journal of Tropical Medicine and Hygiene. 1992b; 47:112–116. [PubMed: 1636877]

Wongsrichanalai C, Dung NT, Trung TN, Wimonwattrawatee T, Sookto P, Heppner DG, Kawamoto F. In vitro susceptibility of *Plasmodium falciparum* isolates in Vietnam to artemisinin derivatives and other antimalarials. Acta Tropica. 1997; 63:151–158. [PubMed: 9088428]

Wongsrichanalai C, Wimonwattrawatee T, Sookto P, Laoboonchai DG, Heppner DG, Kyle DE, Wernsdorfer WH. In vitro sensitivity of *Plasmodium falciparum* to artesunate in Thailand. Bulletin of the World Health Organization. 1999; 77:392–398. [PubMed: 10361756]

Europe PMC Funders Author Manuscripts

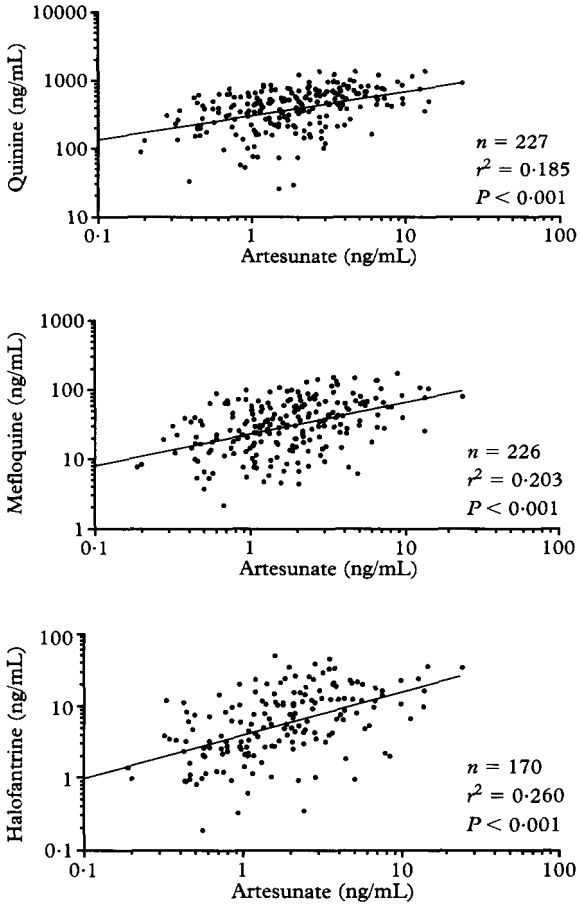

Fig. 1.

Scatter plots of the relationship between the 50% inhibitory concentrations (IC_{50}) against *Plasmodium falciparum in vitro* of artesunate and those of quinine, mefloquine, and halofantrine.

Europe PMC Funders Author Manuscripts

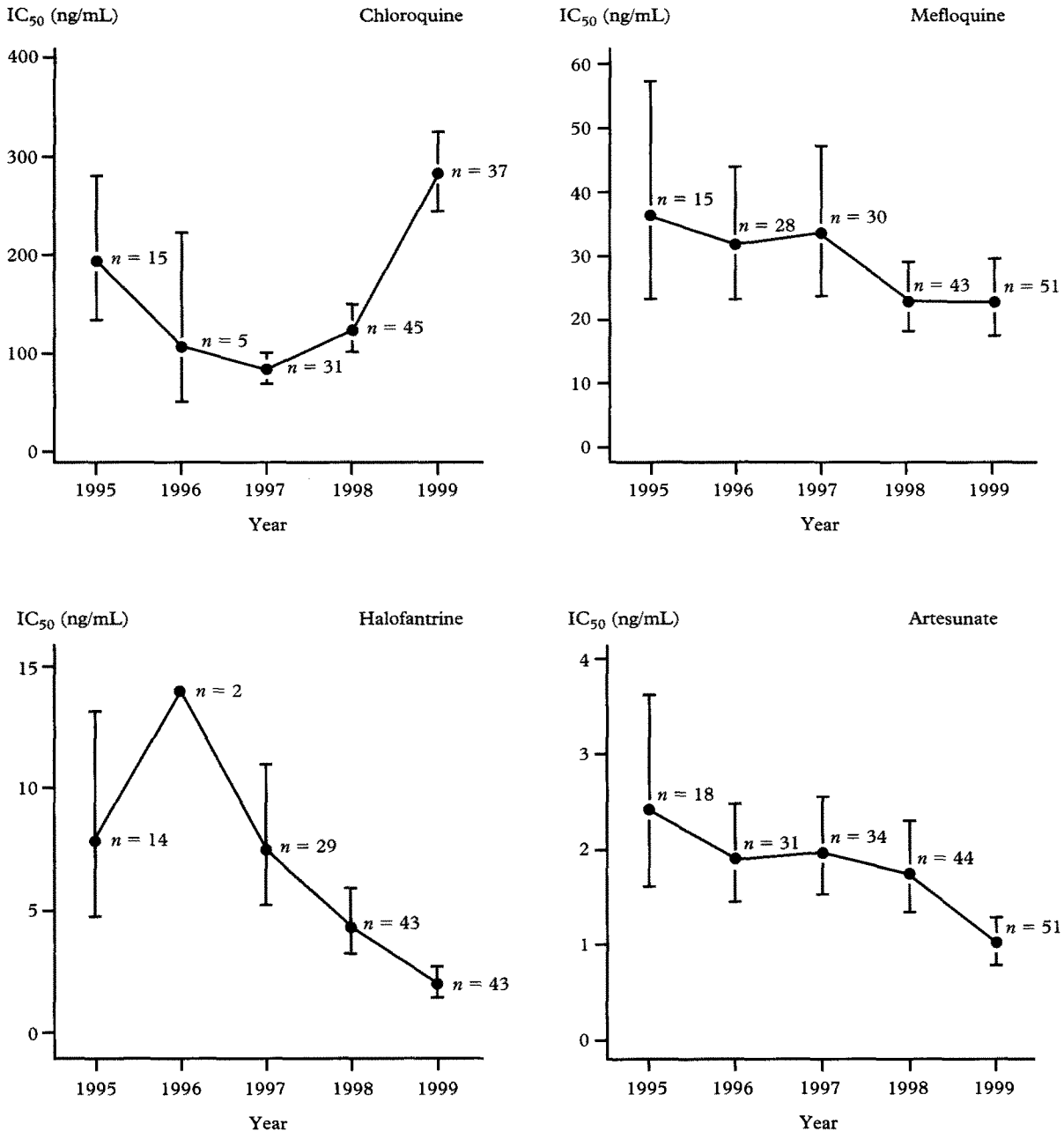

Fig. 2.

Trend of drug susceptibilities *in vitro* of isolates at *Plasmodium falciparum* from primary infections over time. Values are geometric mean 50% inhibitory concentrations (IC_{50}) and 95% confidence intervals.

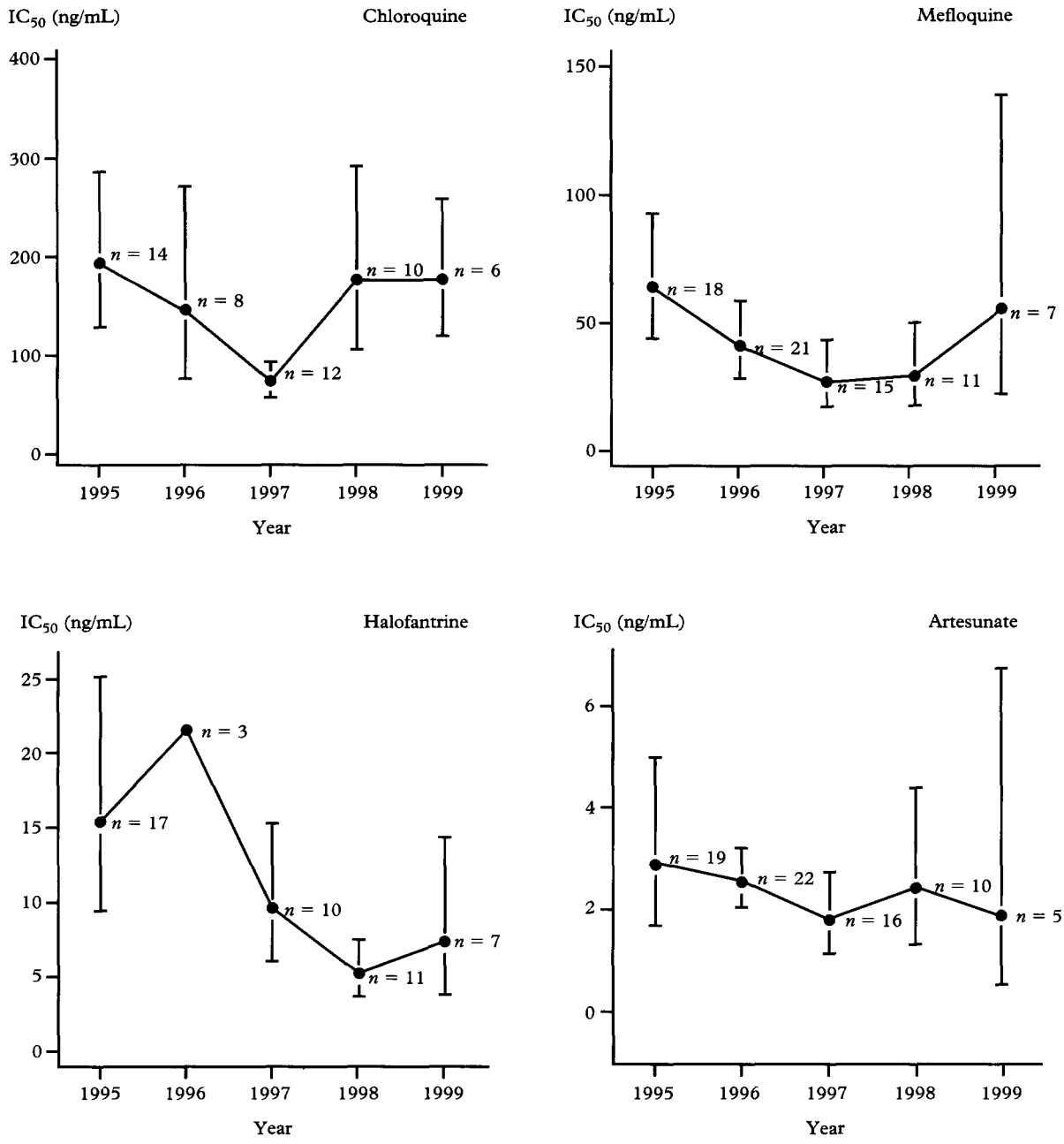

Fig. 3.

Trend of drug of susceptibilities *in vitro* of isolates of *Plasmodium falciparum* from recrudescent infections over time. Values are geometric mean 50% inhibitory concentrations (IC_{50}) and 95% confidence intervals.

Table 1

Numbers of isolates of *Plasmodium falciparum* and clinical and laboratory variables of the patients

	All	Primary infections[a]	Recrudescent infections[b]
Total no. of isolates assayed	268	189	79
No. assayed			
1995	39 (15%)	18 (10%)	21 (27%)
1996	57 (21%)	33 (18%)	24 (30%)
1997	51 (19%)	35 (19%)	16 (20%)
1998	61 (23%)	50 (27%)	11 (14%)
1999	60 (22%)	53 (28%)	7 (9%)
Age (years)[c]	17 (1–65)	18 (1–65)	16 (2–60)
No. of male patients	187 (70%)	128 (68%)	59 (75%)
Parasite density (per/mm^3)[d,e]	35547 (29174–43301)	57108 (48585–67127)	11434 (7178–18214)
No. isolates from Maela camp[f]	236 (88%)	172 (91%)	64 (88%)
No. isolates requiring short term *in vitro* culture[e]	68 (25%)	24 (13%)	44 (56%)
Days in culture before drug assay[c]	4 (2–13)	3 (2–13)	4 (2–8)

[a] No prior history of falciparum malaria within the previous 2 months.

[b] Confirmed history of falciparum malaria in the preceding 42 d.

[c] Median (range in parentheses).

[d] Geometric mean (95% confidence interval in parentheses).

[e] $P < 0{\cdot}001$.

[f] $P = 0{\cdot}04$.

Europe PMC Funders Author Manuscripts

Table 2

Drug sensitivity of the K1 isolate of *Plasmodium falciparum* in 1996 and 1998

	50% Inhibitory concentrations (ng/mL)[a]	
	4 June 1996	**1 June 1998**
Chloroquine	301·5 (196·9–360·4)	215·6 (180–7–261·5)
Quinine	646·8 (479·8–706·1)	610·0(542·4–720·4)
Mefloquine	26·5 (20·8–29·3)	23·5 (17·9–28·8)
Artesunate	5·6 (4·7–6·9)	4·6 (4·0–5·5)
Dihydroartemisinin	3·6 (2·3–3·8)	3·1 (2·9–3·6)
Artemether	3·4 (3·1–7·1)	4·0 (3·7–4·2)

[a]Median values (ranges in parentheses). Five replicates with each drug in 1996, 4 in 1998. No comparison between years was significant by the Mann–Whitney *U* test.

Table 3

Drug sensitivity of isolates of *Plasmodium falciparum* from primary or recrudescent infections

		IC_{50} (ng/mL)[a]		
	No.	**Mean**	**95% CI**	**Range**
Primary infections				
Chloroquine	133	148·5	131·1–168–2	23·4–538·4
Quinine	165	354·1	317·0–395·5	24·6–1326·2
Mefloquine[b]	167	27·1	23·7–30·9	2·4–155·7
Halofantrine[b]	131	4·12	3·4–5·0	0·14–33·0
Lumefantrine	20	32·3	21·7–48·0	2·0–75·6
Artesunate[b]	178	1·62	1·4–1·8	0·19–13·3
Dihydroartemisinin	84	1·22	1·0–1·5	0·15–6·6
Artemether	26	4·83	3·2–7·3	0·39–17·4
Atovaquone[b]	35	0·41	0·26–0·65	0·004–6·1
Recrudescent infections				
Chloroquine	50	140·1	114·6–171·3	45·5–495·8
Quinine	74	431·6	368·1–506·2	51·7–1343·7
Mefloquine[b]	72	41·2	33·8–50·4	6·0–178·1
Halofantrine[b]	48	9·92	7·7–12·8	2·0–50·1
Lumefantrine	12	41·5	30·2–56·9	20·2–87·1
Artesunate[b]	72	2·35	1·9–2·9	0·34–23·4
Dihydroartemisinin	44	1·43	1·1–1·8	0·24–8·1
Artemether	22	5·44	4·2–7·1	1·1–13·1
Atovaquone	5	2·68	0·58–12·5	0·62–14·3

[a] Geometric mean 50% inhibitory concentrations *in vitro*.

[b] Significant difference between primary and recrudescent infection ($P < 0·01$; unpaired *t* test).

Europe PMC Funders Author Manuscripts

Table 4

Correlation between responses *in vitro* of fresh isolates of *Plasmodium falciparum* to artemisinin derivatives and standard antimalarial drugs

Drug pair		No.[a]	r[b]
Mefloquine	Halofantrine	167	0·65[C]
Mefloquine	Quinine	218	0·62[C]
Mefloquine	Artesunate	226	0·45[C]
Mefloquine	Chloroquine	165	−0·03[d]
Artesunate	Halofantrine	170	0·51[C]
Artesunate	Quinine	227	0·43[C]
Artesunate	Chloroquine	172	−0·07[d]
Quinine	Halofantrine	164	0·45[C]

[a] Number of isolates.

[b] Pearson's correlation coefficient.

[C] $P<0·001$.

[d] $P< 0·04$.