In vitro susceptibility of Tanzanian wild isolates of *Plasmodium falciparum* to artemisinin, chloroquine, sulfadoxine/pyrimethamine and mefloquine

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SUMMARY

A 30-h *in vitro* susceptibility test of *Plasmodium falciparum* wild isolates to artemisinin, chloroquine, sulfadoxine/pyrimethamine and mefloquine was performed in Kibaha, Tanzania. A sigmoid E_{max} model was fitted to all data for each isolate and drug combination. Artemisinin and mefloquine exhibited 100% growth inhibition against all isolates tested ($n = 69–74$). The EC_{50} values for artemisinin and mefloquine were 44 and 146 nM respectively. Chloroquine and sulfadoxine/pyrimethamine resistance was 30% and 13% respectively. Susceptibility parameters ($EC_{50, 90, 95}$ and $_{99}$ values and s) varied between compounds and isolates indicating the different sensitivity of *P. falciparum* isolates. No correlation between susceptibility parameters of artemisinin and the other compounds was found. The high *in vitro* activities of artemisinin and mefloquine indicate their potential role for the treatment of multidrug-resistant malaria in Africa.

Key words: *Plasmodium falciparum*, susceptibility, artemisinin, Tanzania.

INTRODUCTION

The emergence of *Plasmodium falciparum* resistant to the standard anti-malarial drugs has led to the use of artemisinin and its derivatives as first-line drugs in Southeast Asia. The compounds have also been registered in some parts of Africa where alternative anti-malarial drugs are available.

With the exception of a few reference strains and isolates of *P. falciparum* (Hassan Alin, Björkman & Ashton, 1990; Hassan Alin *et al*. 1995; Doury *et al*. 1992; Basco & Le Bras, 1993, 1994), the response pattern of artemisinin compounds to parasite heterogeneity in Africa and particularly in Tanzania has not been assessed. Chloroquine is the first-line drug for the treatment of falciparum malaria in Tanzania though with high *in vivo* resistance of up to 59% (Premji, Minjas & Shiff, 1994). The combination of sulfadoxine/pyrimethamine is widely used although sporadic falciparum malaria resistance *in vivo* and a few *in vitro* studies have been reported (Draper *et al*. 1988).

The present study assesses the *in vitro* susceptibility of Tanzanian isolates of *P. falciparum* to artemisinin, chloroquine, sulphadoxine/pyrimethamine and mefloquine. Any *in vitro* cross-resistance patterns among these compounds were also investigated.

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MATERIALS AND METHODS

Study area

The investigation was conducted during July and August 1994, in Kibaha, 40 km northwest of Dar es Salaam, Tanzania. The area is holoendemic for malaria with high transmission in March–June and October–December. Most of the inhabitants are farmers. In one study conducted in 2 villages in the region, high chloroquine-resistance *in vivo* of up to 59% has been reported (Premji *et al*. 1994).

Study population

All symptomatic patients with single infections of falciparum malaria, verified by thick and thin blood films, were identified at the Outpatient Clinics of the Kibaha designated District Hospital. Asexual parasitaemias in excess of 1000 parasites, but less than 80000 parasites/μl blood were considered suitable for testing. Other criteria for inclusion in the study included no history of anti-malarial intake for the present illness. Informed consent was obtained from all patients prior to participation in the study.

Test procedure

Micro *in vitro* tests (WHO, 1987) were performed to assess the susceptibility of *P. falciparum* to artemis-

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Table 1. Mean (95 % CI) parameter estimates of a sigmoid E_{max} model fitted to *in vitro* inhibition tests of artemisinin, chloroquine and Fansidar® (sensitive isolates only) and mefloquine against *Plasmodium falciparum* wild isolates from Kibaha, Tanzania

(EC_{50}, EC_{90}, EC_{95} and EC_{99} are concentrations at which 50, 90, 95 and 99 % growth inhibition occurs. $EC_{90,95}$ and $_{99}$ values were calculated from the model equation using fitted parameter values.)

Drugs	E_{max} (%)	*s*	EC_{50} (nM)	EC_{90} (nM)	EC_{95} (nM)	EC_{99} (nM)
Artemisinin	101	1·0	44	576	1485	13358
(*n* = 69)	(100, 102)	(0·9, 1·1)	(39, 50)	(435, 718)	(1038, 1933)	(7637, 19096)
Chloroquine	100	1·7	51	210	377	1361
(*n* = 50)	(100, 101)	(1·6, 1·9)	(46, 55)	(180, 259)	(293, 465)	(856, 1866)
Fansidar®*	100	1·1	989	9533	21328	133021
(*n* = 64)	(100, 101)	(1·0 1·2)	(758, 1220)	(7323, 11743)	(16162, 26495)	(95950, 170093)
Mefloquine	100	1·9	146	586	985	3456
(*n* = 74)	(99·7, 100)	(1·7, 2)	(130, 163)	(460, 713)	(694, 1276)	(1518, 5394)

* Sulphadoxine/pyrimethamine (ratio 80:1).
n, Number of isolates.

Table 2. Mean (95 % CI) parameter estimates of a sigmoid E_{max} model fitted to *in vitro* inhibition tests of chloroquine and Fansidar® (resistant isolates only) against *Plasmodium falciparum* wild isolates from Kibaha, Tanzania

(EC_{50} values are concentrations at which 50 % growth inhibition occurs.)

Drugs	E_{max} (%)	*s*	EC_{50} (nM)
Chloroquine	76	1·8	212
(*n* = 21)	(68, 83)	(1·5, 2·1)	(161, 262)
Fansidar®*	72	0·6	15297
(*n* = 9)	(64, 80)	(0·5, 0·8)	(7121, 23474)

* Sulfadoxine/pyrimethamine (ratio 80:1).
n, Number of isolates.

inin, chloroquine, sulfadoxine/pyrimethamine and mefloquine. From each patient, 0·1 ml of blood was collected by finger prick and added to 0·9 ml of culture medium. A 50 μl aliquot of the blood medium mixture was pipetted into each well of micro-culture plates pre-dosed with artemisinin, chloroquine, sulfadoxine/pyrimethamine and mefloquine. The final concentrations in the blood medium mixture for artemisinin ranged from 3×10^{-9} to 3×10^{-6} M, for chloroquine from 2×10^{-8} to 128×10^{-8} M, for sulfadoxine/pyrimethamine from 2×10^{-7} to 2×10^{-4} M (ratio 80:1) and for mefloquine from 4×10^{-8} to 256×10^{-8} M. The micro-culture plates were gently agitated, placed in an airtight candle jar and incubated for 30 h at 37·5 °C. After incubation, the contents of the test wells were harvested by removal of the supernatant and the red blood cells deposited on the flat bottom of the wells were transferred to a microscope slide to form thick films, which were stained for 30 min in Giemsa stain. The number of schizonts/200 asexual parasites was counted twice. Experimental runs in which $\geqslant 10$ % of the parasites developed into schizonts in the control wells were considered successful. Chloroquine, sulfadoxine/pyrimethamine and mefloquine plates were obtained from the WHO Regional Office, Manila, the Philippines. Artemisinin plates were kindly provided by Professor Walther Wernsdorfer, Institute for Specific Prophylaxis and Tropical Medicine, University of Vienna, Austria. Schizont growth at $1{\cdot}6\times10^{-7}$ M for chloroquine, $1{\cdot}28\times10^{-6}$ M for mefloquine and 2×10^{-5} M for sulfadoxine/pyrimethamine was considered an indication of resistance (WHO, 1987; W. Wernsdorfer, personal communication).

Data analysis

A sigmoid E_{max} model was fitted to the *in vitro* inhibition data according to:

$$\text{Percentage inhibition} = \frac{E_{max}\times C^{s}}{EC_{50}^{s}+C^{s}},$$

where E_{max} is the maximum growth inhibitory effect, C is the drug concentration in the blood medium mixture, EC_{50} is the drug concentration at which 50 % of the growth inhibition occurs and *s* is the sigmoidicity factor. The equation was applied to inhibition data from the *in vitro* tests using the non-linear regression programme Minim™ (Purves, 1993). Calculations of 90, 95 and 99 % of the maximum growth inhibition ($EC_{90,95}$ and $_{99}$ values) were based on the estimated parameters.

Statistical evaluation

The parameter estimates (EC_{50}, *s* and E_{max}) and EC_{90}, EC_{95} and EC_{99} values of the 4 compounds were

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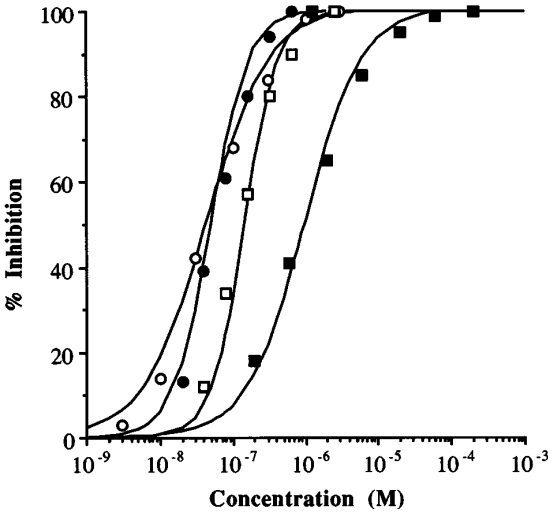

Fig. 1. Growth inhibition of falciparum malaria isolates by artemisinin (○), chloroquine (●) (sensitive isolates only), sulfadoxine/pyrimethamine (■) (sensitive isolates only) and mefloquine (□). Symbols are means of all isolates ($n = 50$–74). Curves were generated with mean parameter estimates taken from Table 1.

compared by one-way ANOVA. Correlations between parameters were tested by linear regression. The level of significance was set to $\leqslant 0{\cdot}05$ in all statistical analysis.

RESULTS

Isolates of falciparum malaria successfully cultured in the tests were 69/102 for artemisinin, 70/105 for chloroquine, 74/105 for mefloquine and 64/98 for sulfadoxine/pyrimethamine. Average parameter estimates (EC_{50}, s and E_{max}) and EC_{90}, EC_{95} and EC_{99} values for the 4 compounds against all isolates are given in Tables 1 and 2. Mean observed data and simulated concentration–effect relationships are shown in Fig. 1.

Artemisinin and mefloquine were both highly effective against all isolates tested, while 30% and 13% of the isolates were, respectively, resistant to chloroquine and sulfadoxine/pyrimethamine. The $EC_{50,90,95}$ and $_{99}$ values for artemisinin, chloroquine (sensitive isolates), mefloquine and sulfadoxine/pyrimethamine (sensitive isolates) were significantly different ($P \leqslant 0{\cdot}001$). The sigmoidicity factor (s) for the 4 compounds also differed ($P \leqslant 0{\cdot}001$).

For the 21 and 9 isolates of falciparum malaria resistant to chloroquine and sulfadoxine/pyrimethamine, respectively, the upper 95% confidence limit of the E_{max} estimate did not reach 100% growth inhibition. The EC_{50} values for chloroquine and sulfadoxine/pyrimethamine were $\leqslant 1{\cdot}6 \times 10^{-7}$ and 2×10^{-5} M respectively for these isolates.

Susceptibility parameters ($EC_{50,90,95}$ and $_{95}$ values and s) for artemisinin did not correlate with those for chloroquine, mefloquine or sulfadoxine/pyrimethamine. Chloroquine correlated neither with mefloquine nor with sulfadoxine/pyrimethamine. I did not find any correlation between mefloquine and sulfadoxine/pyrimethamine.

DISCUSSION

The study investigated the susceptibility of falciparum malaria to artemisinin, chloroquine, sulfadoxine/pyrimethamine and mefloquine in Kibaha, Tanzania.

Artemisinin fully inhibited all isolates of falciparum tested with EC_{50} values ranging from 10 to 109 nM. The high sensitivity of the isolates to the drug confirms previous *in vitro* studies on artemisinin in Africa and in Southeast Asia (Doury *et al.* 1992; Basco & Le Bras, 1993, 1994; Hassan Alin *et al.* 1995).

Chloroquine was effective against 50/70 isolates tested (70%). The EC_{50} values for the sensitive isolates ranged from 13 to 83 nM. In 2 villages in the same region, a chloroquine-resistance *in vivo* of up to 59% was reported (Premji *et al.* 1994). This discrepancy could be due to the sample size of the present study which was smaller compared to that of the other study.

Mefloquine EC_{50} values were in the range of 49 to 363 nM confirming one previous *in vitro* study conducted in the same area (Hassan Alin *et al.* 1995). High sensitivity to the drug has generally been reported in several other studies in Africa (Björkman & Phillips-Howard, 1990), although some studies in West Africa have suggested the occurrence of mefloquine resistance *in vitro* (Brasseur *et al.* 1988; Chippaux *et al.* 1990).

The EC_{50} values for the combination of sulfadoxine and pyrimethamine in the present study was between 107 and 6330 nM for the sensitive isolates. Only 9/73 isolates were found to be resistant. This drug combination is widely used in Tanzania with few earlier *in vitro* studies reported (Draper *et al.* 1988). The high sensitivity of falciparum malaria to sulfadoxine and pyrimethamine combination still supports the use of this drug combination as a first-line treatment for chloroquine-resistant infections. However, continuous *in vitro* monitoring and surveillance are necessary.

The coefficient of variation (CV) of the EC_{50} values for artemisinin, chloroquine (sensitive isolates), sulfadoxine/pyrimethamine (sensitive isolates) and mefloquine were respectively 51, 32, 92 and 49%, reflecting the different susceptibility of the isolates. Median EC_{50} values increased in the order: artemisinin < chloroquine < mefloquine < sulfadoxine and pyrimethamine combination.

No correlation between parameter estimates (EC_{50} and s) and EC_{90}, EC_{95} and EC_{99} values for the 4 compounds were found. The results of the present study agree with one previous study in the Kibaha area in which no correlation was shown between

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parameter estimates of artemisinin and mefloquine (Hassan Alin *et al*. 1995). A negative correlation of artemisinin and chloroquine has been shown in African isolates (Doury *et al*. 1992). In contrast, the present study did not reveal any correlation between the parameter estimates of artemisinin and chloroquine.

The chloroquine resistance was moderate, while sulfadoxine/pyrimethamine resistance was mild. The sulfadoxine and pyrimethamine combination is widely used in Tanzania. Continuous monitoring of the sensitivity of falciparum malaria to the compound is necessary.

In conclusion, the results of the study show the high sensitivity of falciparum malaria isolates to artemisinin and mefloquine with artemisinin having the lowest EC_{50} value compared to the 3 compounds.

Artemisinin and its derivatives are now the first-line drugs in several countries in Southeast Asia and are also available in some African countries. There are concerns that the indiscriminate use of the compounds may result in a rapid development of resistance. Rational drug use with continuous *in vitro* monitoring should be applied.

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