In vitro susceptibility of Gabonese wild isolates of *Plasmodium falciparum* to artemether, and comparison with chloroquine, quinine, halofantrine and amodiaquine

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SUMMARY

The *in vitro* activity of artemether against 63 African isolates of *Plasmodium falciparum* from Libreville, Gabon was evaluated using an isotopic drug susceptibility semi-microtest. The 50% inhibitory concentration (IC_{50}) values for artemether were in a narrow range from 0·8 to 34·8 nM (mean IC_{50} 5·0 nM) and the 95% confidence interval (CI95%) was 3·6–6·3 nM. *In vitro* decreased susceptibility or resistance were observed with artemether (14%), to chloroquine (90%), to quinine (32%). Isolate susceptibility to amodiaquine and halofantrine was high i.e. 100% and 98%, respectively. There was a significant positive correlation between responses to artemether and amodiaquine ($r^2 = 0{\cdot}45$, $P < 0{\cdot}001$), artemether and chloroquine ($r^2 = 0{\cdot}36$, $P < 0{\cdot}001$), artemether and quinine ($r^2 = 0{\cdot}31$, $P < 0{\cdot}001$), and artemether and halofantrine ($r^2 = 0{\cdot}19$, $P < 0{\cdot}01$). Positive correlation between these drugs suggests *in vitro* cross-resistance or at least common features in drug uptake and/or mode of action or resistance.

Key words: malaria, *Plasmodium falciparum*, artemether, chloroquine resistance, Gabon.

INTRODUCTION

In the absence of effective and practical preventive measures, the only current options for reducing the morbidity and mortality of malaria, especially in Africa, are chemoprophylaxis and chemotherapy. The emergence of *Plasmodium falciparum* resistance to the standard anti-malarial drugs has led to the use of artemisinin and its derivatives as first-line drugs in southeast Asia. Artemether (an oil-soluble artemisinin-derivative) has also been registered in some parts of Africa and especially in Gabon. With the exception of a few reference strains and isolates of *P. falciparum* (Alin, Bjorkman & Ashton, 1990; Bustos, Gay & Diquet, 1994 (Philippines); Basco & Le Bras, 1994 (Cambodia)), the *in vitro* response pattern of artemether to wild isolates in Africa (Basco & Le Bras, 1993; Pradines *et al.* 1998 (Senegal)) particularly in Gabon has not been assessed. The artemisinin derivatives, including artemether, which act more rapidly against *P. falciparum* than other anti-malarial drugs, have proven efficacy in the treatment of severe malaria in adults in Asia (Hien *et al.* 1996; Vinh *et al.* 1997) and in children with cerebral malaria in Africa (Boele van Hensbroek *et al.* 1996; Murphy *et al.* 1997).

The present study assesses the *in vitro* susceptibility of Gabonese wild isolates of *P. falciparum* to artemether, chloroquine, quinine, halofantrine and amodiaquine. Any *in vitro* cross-resistance patterns among these compounds were also investigated.

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MATERIALS AND METHODS

Isolates of P. falciparum

Between April and July 1997 we analysed the drug sensitivity patterns of 63 fresh *P. falciparum* isolates obtained from hospitalized children (6 months–15 years old) with uncomplicated or severe malaria from Libreville (Gabon). Venous blood was collected before treatment in Vacutainer^R ACD tubes (Becton Dickinson, Rutherford, NJ, USA) and transported at 4 °C to Marseille within 96 h. Thin blood smears were stained using an RAL^R kit (Réactifs RAL, Paris, France) and examined to determine parasite density and to confirm monoinfection by *P. falciparum*. Samples with parasitaemia ranging from 0·03 to 7·8% were used to test drug sensitivity. Parasitized erythrocytes were washed 3 times in

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Table 1. *In vitro* activities of artemether, amodiaquine, halofantrine, chloroquine and quinine against Gabonese wild isolates of *Plasmodium falciparum*

(Values are the geometric mean 50 % inhibitory concentrations (IC_{50}). Threshold IC_{50} values for *in vitro* reduced susceptibility or resistance to artemether, amodiaquine, halofantrine, chloroquine and quinine are > 10·5 nM, > 80 nM, > 6 nM, > 100 nM and > 500 nM, respectively.)

Drugs	Mean IC_{50} (nM)	95 % confidence intervals	Resistant isolates (%)
Artemether	5·0	3·6–6·3	14
Amodiaquine	10·3	8·5–12·2	0
Halofantrine	1·5	1·1–1·9	2
Chloroquine	249	203–295	90
Quinine	275	215–335	32

RPMI-1640 medium (Gibco BRL, Paisley, UK). If parasitaemia exceeded 0·8 %, infected erythrocytes were diluted to 0·5–0·8 % with uninfected erythrocytes and resuspended in culture medium to a haematocrit of 1·5 %. Susceptibility to the different anti-malarial drugs was determined after suspension in RPMI-1640 medium supplemented with 10 % human serum (pooled from different A^+ or AB, non-immune out of the area of malaria endemicity donors) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$.

Drugs

Artemether was obtained from Rhône Poulenc Rorer Doma (Antony, France), chloroquine diphosphate, quinine hydrochloride and amodiaquine dihydrochloride from Sigma Chemical (St Louis, MO, USA), and halofantrine hydrochloride from Smith Kline & French (Paris, France). Stock solutions were prepared in methanol for artemether, quinine and halofantrine in sterile distilled water for chloroquine diphosphate, and amodiaquine dihydrochloride (there was no methanolic cytotoxicity on parasite growth and no evidence of precipitation of anti-malarials when dilutions were made in water). Two-fold serial dilutions were prepared in sterile distilled water. Final concentrations, ranged from 0·8 to 100 nM for artemether, 25 to 3200 nM for chloroquine, 50 to 3200 nM for quinine, 3·1 to 400 nM for amodiaquine, and 0·25 to 32 nM for halofantrine were distributed in triplicate into Falcon 96-well flat-bottomed plates.

The chloroquine-susceptible D6 *P. falciparum* clone (Sierra Leone) and the chloroquine-resistant W2 clone (Indochina) were used as references to test each batch of plates. Reference clones were maintained in continuous culture and twice synchronized with sorbitol.

In vitro *assay*

For *in vitro* isotopic microtests, the suspension of parasitized erythrocytes was distributed under 200 μl/well in 96-well plates pre-dosed with anti-malarial agents. Parasite growth was assessed by adding 1 μCi of [^{3}H]hypoxanthine with a specific activity 14·1 Ci/mmol (NEN Products, Dreiech, Germany) to each well. Plates were incubated for 42 h at 37 °C in an atmosphere of 10 % O_2, 6 % CO_2, 84 % N_2, and a humidity of 95 % (optimum conditions in our laboratory). Immediately after incubation the plates were frozen then thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter™ GF/B, Packard Instrument Company, Meriden, CT, USA) and washed using a cell harvester (FilterMate™ Cell Harvester, Packard). Filter microplates were dried and 25 μl of scintillation cocktail (Microscint™ O, Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count™, Packard).

The 50 % inhibitory concentration (IC_{50}) i.e. the drug concentration corresponding to 50 % of the uptake of [^{3}H]hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were expressed as the geometric mean IC_{50} and 95 % confidence intervals (95 % CI) were calculated.

Isolates were considered as chloroquine-resistant if IC_{50} was greater than 100 nM. Cut-off values for resistance to artemether, amodiaquine, quinine and halofantrine were 10·5 nM (Pradines *et al*. 1998), 80 nM, 500 nM and 6 nM, respectively. The *in vitro* threshold value of anti-malarials has been defined statistically (> 2 S.D. above the mean). Only *in vitro* resistance to chloroquine, evaluated statistically, has been confirmed by correlation with *in vivo* therapeutic effectiveness.

Assessment of investigated anti-malarial cross-resistance was estimated by Pearson correlation coefficient (r) and coefficient of determination (r^2).

RESULTS

Average parameter estimates for the 5 compounds against all isolates are given in Table 1. The 63 IC_{50} values for artemether were in a narrow range from 0·8 to 34·8 nM (mean IC_{50} = 5·0 nM) and CI95 % was 3·6–6·3 nM. Amodiaquine, halofantrine and artemether were both highly effective against Gabonese isolates, while 90 % of the isolates were *in vitro* resistant to chloroquine and 32 % showed *in vitro* decreased susceptibility to quinine.

A significant positive correlation, suggesting *in vitro* cross-resistance among these drugs was found between artemether and amodiaquine (r^2 = 0·45,

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Table 2. Correlation of *in vitro* responses of 62 Gabonese isolates of *Plasmodium falciparum* to artemether, amodiaquine, halofantrine, chloroquine and quinine

(r = Pearson correlation coefficient ($n = 62$) (*$n = 44$), and r^2 = coefficient of determination.)

Drug pair		r	r^2	P
Artemether	Amodiaquine	+0·68	0·45	< 0·001
Artemether	Chloroquine	+0·60	0·36	< 0·001
Artemether	Quinine	+0·56	0·31	< 0·001
Artemether	Halofantrine*	+0·44	0·19	< 0·01
Chloroquine	Quinine	+0·63	0·40	< 0·001
Chloroquine	Amodiaquine	+0·60	0·36	< 0·001
Chloroquine	Halofantrine*	+0·47	0·22	< 0·01
Quinine	Halofantrine*	+0·75	0·56	< 0·001
Quinine	Amodiaquine	+0·60	0·36	< 0·001
Amodiaquine	Halofantrine*	+0·68	0·46	< 0·001

$P < 0·001$), chloroquine ($r^2 = 0·36$, $P < 0·001$), quinine ($r^2 = 0·31$, $P < 0·001$), and halofantrine ($r^2 = 0·19$, $P < 0·01$) (Table 2).

DISCUSSION

Artemether is highly effective against wild isolates in Gabon, where 90% of the isolates were resistant to chloroquine *in vitro* and 32% showed decreased susceptibility *in vitro*. An *in vitro* study, conducted in Lambarene, Gabon, showed that all isolates tested for response to chloroquine were resistant (Winkler *et al*. 1994). *In vivo* work, performed in the same place, showed that 65% of patients had recrudescent malaria or persistent parasitaemia after chloroquine treatment (Kremsner *et al*. 1993).

There are conflicting reports on artemisinin derivative activities against chloroquine-resistant and chloroquine-susceptible strains of *P. falciparum*. Previous studies in Central and West Africa (Basco & Le Bras, 1993) and in Cameroon (Ringwald, Bickii & Basco, 1996) showed that susceptibility to artemether was inversely related to susceptibility to chloroquine. The present study showed a weakly positive significant correlation between artemether and chloroquine ($r^2 = 0·36$, $P < 0·001$), reported by other workers using field isolates or culture-adapted strains of *P. falciparum* (Hassan Alin *et al*. 1995), in contrast with previous findings indicating negative correlation between responses to artemisinin and chloroquine (Doury *et al*. 1992; Basco & Le Bras, 1993) or no correlations (Hassan Alin, 1997).

Positive correlation between artemether and quinine ($r^2 = 0·31$, $P < 0·001$) was shown, confirming data reported in a previous study (Bustos *et al*. 1994). However, the artemisinin derivatives are satisfactory alternatives to quinine for the treatment of severe malaria in adults (Hien, 1996) or cerebral malaria in children (Boele van Hensbroek *et al*. 1996; Thanh Phuong *et al*. 1997). However, significant positive correlation may suggest *in vitro* cross-resistance or at least *in vitro* cross-susceptibility, which is not necessarily predictive of cross-resistance *in vivo*.

We noted positive significant correlations between the responses to artemether and amodiaquine ($r^2 = 0·45$, $P < 0·001$), halofantrine ($r^2 = 0·19$, $P < 0·01$) and also, between all the anti-malarial drugs investigated (chloroquine, quinine, halofantrine and amodiaquine).

The high activity of artemether leads us to believe that artemether may be an important alternative drug for the treatment of chloroquine-resistant malaria, despite the positive correlations of *P. falciparum* isolates between responses to artemether, amodiaquine, quinine, halofantrine and chloroquine, suggesting *in vitro* cross-resistance or at least common features in drug uptake and/or mode of action or resistance. However, *in vitro* cross-resistance reinforces the idea that novel anti-malarials should not be deployed for monotherapy. There is an urgent need to find a rational partner compound with which artemether can be administered in order to prolong its potentially useful life, and this, before and not after resistance has begun to emerge. Furthermore, the relatively short half-lives of artemether and artemisinin derivatives may be one of the factors responsible for the poor radical cure rate of falciparum malaria (de Vries & Dien, 1996; Mordi *et al*. 1997). Potentiation between pyronaridine and artemether was observed against rodent parasites (Peters & Robinson, 1997). Several recent studies have confirmed that combination of artemisinin-related compounds with mefloquine is highly effective even against mefloquine-resistant *P. falciparum* (Nosten *et al*. 1994; Karbwang *et al*. 1995; Price *et al*. 1995; Na-Bangchang *et al*. 1997, Looareesuwan *et al*. 1997). Combination of artesunate with tetracycline is also effective in the treatment of uncomplicated falciparum malaria and may provide a useful alternative to other treatment regimens (Duarte *et al*. 1996). Combination of artemether with benflumetol may be an alternative treatment. Clinical works are in progress. There was no significant difference in therapeutic response parameters between artemether-benflumetol and artesunate-mefloquine (Van Vugt *et al*. 1998). It will be important to continuously monitor the response of parasites to artemether and other anti-malarial drugs in different geographical regions.

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