African Journal of Biotechnology Vol. 7 (8), pp. 936-940, 17 April, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

*Full Length Research Paper*

# Influence of the asexual parasite biomass on *in vitro* susceptibility of *Plasmodium falciparum* to antimalarial drugs in Abidjan

**Bla Kouakou Brice[1], Yavo William[2], Ouattara Lacinan[1], Basco Leonardo[3] and Djaman Allico Joseph[1, 4]***

[1]Laboratoire de Pharmacodynamie-biochimique, Université de Cocody, 22 BP 582 Abidjan 22, Côte d'Ivoire.
[2]Laboratoire de microbiologie de l'Institut National de Santé Publique (INSP), BP V47 Abidjan, Côte d'Ivoire.
[3]Unité de Recherche Paludologie Afro-tropicale, Institut de recherche pour le développement (IRD) – Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), BP 288 Yaoundé, Cameroun.
[4]Département de Biochimie, Institut Pasteur de Côte d'Ivoire, 01 BP 490 Abidjan, Côte d'Ivoire.

Accepted 6 March, 2008

**The *in vitro* activities of artemisinin, dihydroartemisinin (the biologically active metabolite of artemisinin derivatives), chloroquine and pyronaridine were assessed in 32 isolates of *Plasmodium falciparum* from Abobo in the northern of Abidjan district (Côte d'Ivoire) using a test based on the standard microtechnique recommended by the World Health Organization (WHO). The parasites densities were ranged between 8,000 and 540,000 rings/µl of blood. The geometric means 50% inhibitory concentration ($GMIC_{50}$) values for chloroquine, pyronaridine and artemisinin were 145.5 nM (95% confidence interval (CI) =65-226 nM), 17.69 nM (95% CI=9.1-26.3 nM) and 5.72 nM (95% CI=2.3-9.1 nM), respectively. Dihydroartemisinin was the most potent drug against chloroquine-sensitive and chloroquine-resistant isolates with a geometric mean of 2.72 nM. There was no correlation between the parasite densities and the responses to chloroquine ($r^2$=0.01, p<0.5), pyronaridine ($r^2$=0.13, p<0.05), artemisinin ($r^2$=0.13, p<0.05) and dihydroartemisinin ($r^2$=0.07, p<0.1).**

**Key words:** Artemisinin, chloroquine, dihydroartemisinin, *in vitro* test, parasite biomass, pyronaridine.

## INTRODUCTION

Malaria continues to be a major cause of morbidity and mortality in most tropical countries. In Africa alone 140 - 280 millions people suffer from malaria each year and more than one million die (WHO, 1996). The world malaria situation is aggravated by the fact that an increased prevalence of drug-resistant strains of *Plasmodium falciparum* continues to reduce the effectiveness of most known anti-malarial (White, 1992). To plan and improve local malaria control programs, the susceptibility to antimalarial drugs of the local parasite population should be monitored. This can be done by *in vivo* and *in vitro* testing. The wide spread availability of cheap and effective antimalarial drugs, particularly chloroquine and sulfadoxine - pyrimethamine, has undoubtedly limited both morbidity and mortality, but it has also encouraged the development and spread of resistance. This is a catastrophe for poor tropical countries, which cannot afford more expensive alternative antimalarial drugs. Mortality is already rising (Trape et al., 1998). In Côte d'Ivoire, malaria is the main reason for consultations at health facilities all year round, with a peak between September and December. In 2000, malaria represented between 30 and 40% of the total consultations in the south of Côte d'Ivoire (Yavo et al., 2002). Several *in vitro* sensitivity test system have been developed and applied to sensitivity monitoring of *P. falciparum* in endemic areas. These include traditional *in vitro* tests based on the measurement of effect of drugs on the growth and development of malaria parasites, i.e., schizont maturation or growth inhibition (Rieckmann et al., 1978; WHO, 1990), incorporation of radiolabeled precursor

*Corresponding author. E-mail: djamanj@yahoo.fr. Tél : +225 22 48 10 00 poste 6807.

(Desjardins et al., 1979), enzymatic activity of parasite lactate deshydrogenase (pLDH) (Makler and Hinrichs, 1993) or histidine-rich protein II (HRP II) (Noedl et al., 2002).

More usually, the exposure of a parasite population to sub-inhibitory antimalarial drug concentrations provides the selective pressure to resistance. Whereas a highly resistant mutant (relative to the distribution of susceptibility in sensitive parasite) may survive maximum blood concentrations of the antimalarial drug and thus emerge from the initial biomass, mutants with lesser reducing in susceptibility will still be eliminated. They will only be selected if the initial treatment is inadequate and blood levels are low, or if they arise in subsequent generations. But the numbers of parasites in each succeeding generation after drug treatment starts in orders magnitudes lower than that in the preceding generation; this is an infrequent process (White, 1999). Thus the chance of a drug-resistant mutant malaria parasite being selected by drug depends on the number of parasites, the mutation frequencies, the drug susceptibility and fitness of the mutants. The principal effect of antimalarial drug is to inhibit parasite multiplication (by arresting development). In theory, the untreated infection can multiply at a maximum rate given by the average number of viable merozoites per mature schizont. The main purpose of this study was to explore the effect of parasitemia on *in vitro* susceptibility of *P. falciparum* isolates to chloroquine, artemisinin, dihydroartemisinin and pyronaridine.

## MATERIELS AND METHODS

### Study area

The study was carried out between April to December, 2006 in Abobo, situated in the northern of Abidjan district at 15 km from the center of the capital city. Malaria is hyperendemic with seasonal transmission. The most common vectors were *Anopheles gambiae* and *A. funestus*. Patients aged between 2 to 45 years were recruited at El Rapha and Anokoi Kouté health centres. Informed consent was obtained from the patients or guardian accompanying the sick children. The study was approved by the Ivorian National Ethics Committee.

### Isolates of *P. falciparum*

During the study period, 41 isolates were collected, before treatment, in Vacutainer EDTA tubes (Terumo Europe N.V.,Leuven, Belgium) and transported at 4°C to the microbiology laboratory of National Institute of Public Health (NIPH) situated in Adjamé (13 km from Abobo) within 6 h, if parasite density was ≥4,000 rings/µl of blood. Giemsa-stained thin blood smears were examinated to determine parasite densities and to confirm *P. falciparum* mono-infections. When the parasitemia was between 4,000 and 10,000 rings/µl, samples were used directly in the drug sensitivity test. If parasites densities exceeded 10,000 rings/µl, samples were diluted with uninfected erythrocytes to obtain initial parasitemia between 0.5 and 1%. In the study, asexual parasite densities were ranged between 8,000 and 540,000 rings/µl of blood. Patients were treated by halofantrine, lumefantrine-artemether, amodiaquine-artesunate or quinine following the recommended therapeutic protocols.

### *In vitro* assay

The test compounds were obtained from the following sources: artemisinin (Aldrich, France), dihydroartemisinin and pyronaridine (TDR/WHO Drug Discovery Research) and chloroquine (Sanofi-Adventis, France). Stock solutions of artemisinin and dihydroartemisinin were prepared in 70% methanol. Stock solutions of pyronaridine and chloroquine were prepared in sterile distilled water. The world Health Organization (WHO) microtest technique was used and the inhibition of schizont maturation was measured microscopically (WHO, 1990, unpublished data). Infected erythrocytes were washed three times in RPMI 1640 medium and suspended in RPMI 1640 plus 10% human serum, 25 mM HEPES, and 25 mM $NaHCO_3$ at a hematocrit of 1.5%. Fifty microliters of the blood-medium mixture were pipetted in each well of the predosed 96-well tissue culture plates and incubated at 37°C in candle jars for 42 h according to standard methodology. Final concentrations ranging from 12.5 to 1600 nM for chloroquine, 0.5 to 64 nM for artemisinin and dihydroartemisinin and 1.25 to 160 nM for pyronaridine were distributed in triplicate into plates. The cut-off values for *in vitro* resistance were the following: chloroquine, 100 nM (Le Bras and Ringwald, 1990), artemisinin and dihydroartemisinin, 10 nM (Pradines et al., 1999) and pyronaridine, 15 nM (Pradines et al., 1998). After incubation parasites were harvested and Giemsa-stained thick blood films were prepared. The number of mature schizonts was counted per 200 asexual parasites. Isolates with less than 20% of mature schizonts in the control well were excluded. The results were expressed as 50% as inhibitory concentration values ($IC_{50}$) determinated by log-probit graphs.

### Statistical analysis

The $IC_{50}$s were expressed as the geometric mean and the 95% confidence intervals. Correlation coefficients (r) and coefficients of determination ($r^2$) between the $IC_{50}$s of different drugs and parasite densities were calculated by Spearman rank correlation. The level of significant was set at 0.05.

## RESULTS AND DISCUSSION

Thirty-two (78%) out of the 41 collected isolates were cultured finally; nine (22%) samples had parasites densities lower than 4,000 rings/µl and had been excluded. The following proportions of isolates with successfully cultured for each drug were: 32 of 32 for chloroquine, 27 of 32 for pyronaridine, 25 of 32 for artemisinin, and 28 of 32 for dihydroartemisinin. Average parameter estimates for the 4 drugs against the *P. falciparum* isolates parasites are given in Table 1. The *in vitro* resistance to chloroquine was 56.25%. Thirty-six percent (36%) and 48% of the tested isolates showed *in vitro* resistance to artemisinin and pyronaridine, respectively. Only one (3.6%) parasite was resistant to dihydroartemisinin. Correlations of *in vitro* responses of isolates to antimalarial drugs and asexual parasites densities calculated with coefficient of correlation (r) and coefficients of determination ($r^2$) are given in Table 2. Coefficients of determination, $r^2$, ranged from 0.13 top 0.07. For all drugs tested, no correlation was identified between *in vitro* responses and parasite densities of samples.

This study reports the evaluation of the *in vitro* susceptibility of 4 antimalarial drugs against 32 isolates of

**Table 1.** *In vitro* susceptibilities and prevalence of resistance of *Plasmodium falciparum* from Abidjan.

| Drug | No. Isolates tested | Geometric mean $IC_{50}$ | 95% confidence interval | Rate of resistance |
|---|---|---|---|---|
| Chloroquine | 32 | 145.5 nM | 65-226 nM | 56.25 (18/32) |
| Pyronaridine | 27 | 17.69 nM | 9.1-26.3 nM | 48 (13/27) |
| Artemisinin | 25 | 5.72 nM | 2.3-9.1 nM | 36 (9/25) |
| Dihydroartemisinin | 28 | 2.72 nM | 1.45-3.99 nM | 3.6 (1/28) |

**Table 2.** Correlation between *in vitro* responses of *Plasmodium falciparum* to antimalarial drugs and asexual parasite densities from Abidjan

| No. of isolates | Parasite density (rings/µl) | *In vitro* response | | | |
|---|---|---|---|---|---|
| | | Chloroquine | Pyronaridine | Artemisinin | Dihydroartemisinin |
| 1185 | 60,000 | R | S | S | S |
| 1194 | 12,000 | R | R | S | S |
| 1342 | 9,000 | S | S | S | S |
| 1552 | 90,000 | S | S | S | S |
| 4034 | 16,000 | R | ND | ND | S |
| 4055 | 239,000 | S | ND | ND | ND |
| 4083 | 28,000 | S | ND | ND | S |
| 4102 | 10,000 | S | ND | ND | S |
| 4136 | 36,000 | R | ND | ND | ND |
| $AK_{01}$ | 18,000 | R | R | S | S |
| 7527 | 464,000 | S | S | S | S |
| 7674 | 9,000 | S | S | R | S |
| 7667 | 10,000 | R | R | S | S |
| 8497 | 47,000 | R | S | R | S |
| 8509 | 346,000 | S | S | ND | S |
| 8549 | 59,000 | S | S | S | S |
| 8550 | 116,000 | R | R | S | S |
| 605/07 | 228,000 | S | R | ND | S |
| 612/07 | 8,000 | R | R | R | S |
| $AK_{06}$ | 138,000 | R | R | S | S |
| $AK_{07}$ | 205,000 | R | R | R | S |
| $AK_{08}$ | 20,000 | R | S | R | S |
| $AK_{09}$ | 307,000 | R | R | S | S |
| $AK_{10}$ | 266,000 | R | S | S | S |
| $AK_{11}$ | 52,000 | S | S | S | S |
| $AK_{12}$ | 540,000 | R | R | S | S |
| $AK_{13}$ | 199,000 | R | R | R | S |
| $AK_{14}$ | 30,000 | R | R | R | S |
| $AK_{15}$ | 13,000 | S | R | R | R |
| $AK_{16}$ | 17,000 | R | S | R | S |
| $AK_{17}$ | 110,000 | S | S | S | S |
| $AK_{18}$ | 69,000 | S | S | S | S |
| r | | 0.11 | -0.36 | -0.36 | -0.28 |
| $r^2$ | | 0.01 | 0.13 | 0.13 | 0.07 |
| p | | <0.5 | <0.05 | <0.05 | <0.1 |

S =Sensitive isolates, R = resistant isolates, ND = undeterminated, r = correlation coefficient, $r^2$ = coefficient of determination.

*P. falciparum* in Abidjan. Chloroquine resistance was 56.25%. Chloroquine was the most commonly used antimalarial drug in self treatment in rural or areas of urban of Côte d'Ivoire (Henry et al., 1998). Chloroquine is a cheap, safe, and universally used antimalarial. Initial observations of chloroquine resistance were reported in the early 1960s from South America and South-east Asia (Wernsdorfer and Payne, 1991). Increasing pressure to use chloroquine would probably induce chloroquine resistance. This chloroquine resistance is linked with a diminution of the susceptibility to pyronaridine and artemisinin. Forty-eight and 36% of the isolates tested in our study have $IC_{50}$s higher than the defined cutoff of 15 nM and 10 nM for pyronaridine and artemisinin, respectively. One of the reasons for such decreased susceptibility could be their structural unstably.

Artemisinin is a sesquiterpene lactone characterized by the presence of an endoperoxide that is associated with antimalarial activity (Gay et al., 1994). 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 ether, ester or other substituents to the hydroxyl group of dihydroartemisinin. Pyronaridine is a substituted 1-aza-acridine with a ring system similar to that of mepacrine and also a substituted 1,5-naphthyridine. Its side-chain is structurally similar to the amodiaquine whose activity showed a significant reduction in our laboratory (Yavo et al., 2007, unpublished data). One out of 32 isolates of *P. falciparum* was resistant to dihydroartemisinin. Only 3.6% of parasites developed resistance to that new antimalarial drug. These data confirmed dihydroarte-misinin as a highly active metabolite of artemisinin derivatives. It is used in artemisinin-based combination therapies (ACTs) (Kinderman et al., 2007).

White (1999) showed the effect of initial parasite biomass in determining the therapeutic response with reduced drug sensitivity. Data obtained in our study showed any correlation between *in vitro* responses of *P. falciparum* isolates to antimalarial drugs and the parasite densities. The lower parasitemia was the sample coded 612/07 with 8,000 rings/µl of blood. Isolate 612/07 given the following responses: resistant to chloroquine ($IC_{50}$=650 nM), artemisinin ($IC_{50}$=20 nM) and pyronaridine ($IC_{50}$=56 nM). Moreover it was sensitive to dihydro-artemisinin ($IC_{50}$= 6 nM). Three out of 4 compounds were not active against isolate 612/07. The higher parasitemia was the sample coded $AK_{12}$ with 540,000 rings/µl of blood. This isolate given the following responses: resistant to chloroquine ($IC_{50}$=280 nM) and pyronaridine ($IC_{50}$=27.5 nM). Moreover it was sensitive to artemisinin ($IC_{50}$= 6 nM) and dihydroartemisinin ($IC_{50}$=7 nM). The correlation data between *in vitro* responses and parasite densities were the following: for chloroquine ($r^2$=0.01, $p<0.5$), for pyronaridine ($r^2$=0.13, $p<0.05$), for artemisinin ($r^2$=0.13, $p<0.05$), and for dihydroartemisinin ($r^2$=0.07, $p<0.1$) Table 2.

However, in acute malaria, in adult, asexual parasites densities in the body are usually between $10^9$ and $10^{13}$ (White, 1999). This corresponds to parasitemia between 0.001 and 10%. Assuming a random distribution of mutants, a patient with 1% parasiteamia is therefore 1000 times more likely to harbour a drug resistant mutant than a patient with 0.001% parasitemia (Paul et al., 1995). But this patient is less likely to have significant immunity and is more likely to receive antimalarial drug treatment, so the chance that a drug-resistant parasite will be selected even greater than 1000-fold more than that for patient with the lower parasitemia.

## ACKNOWLEDGMENTS

We are grateful for the hospitably and generous collaboration of El Rapha and Anokoi Kouté Health centres. We thank also the parents of patients included in this study for collaboration.

### REFERENCES

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD (1979). Quantitative assessment of antimalarial activity *in vitro* by semi automated microdilution technique. Antimicrob. Agents Chemother. 16: 710-718.

Gay F, Ciceron L, Litaudon M, Bustos MD, Astgneau P, Diquet B, Danis M, Gentilini M (1994). *In vitro* resistance of *Plasmodium falciparum* ginghaosu derivatives in West Africa. Lancet. 343: 850-851.

Henry MC, Koné M, Guillet P, Mouchet J (1998). Chloroquine resistance and malaria control in Ivory Coast. Cah. Sante. 8: 287-291.

Kinderman JM, Vandenbergh D, Vreeke E, Olliaro P, D'Altilia JP (2007). Estimating antimalarial drugs consumption in Africa before the switch to artemisinin-based combination therapies (ACTs). Mal. J. 6: 91.

Le Bras J, Ringwald P (1990). *Plasmodium falciparum* chemo-resistance: the situation in Africa in 1989. Med. Trop. 50: 161-162

Makler MT, Hinrichs DJ (1993). Measurement of the lactate deshydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am. J. Trop. Med. Hyg. 48: 205-210.

Noedl H, Wernsdorfer WH, Miller RES, Wongsrichanalai C (2002). Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. Antimicrob. Agents Chemother. 46: 1658-1664.

Paul REL, Packer MJ, Walmsley M, Lagog M, Ranford-Cartwright LC, Paru R, Day KP (1995). Mating patterns in malaria parasite populations of Papua New Guinea. Science, 269: 1709-1711.

Pradines BC, Tall A, Parzy D, Spiegel A, Fusai A, Hienne R, Doury JC (1998). *In vitro* activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial agents. J. Antimicrob. Chemother. 42: 333-339.

Pradines BC, Rogier T, Fusai A, Tall A, Trape JF, Doury JC (1999). *In vitro* activity of artemether against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs. Am. J. Trop. Med. Hyg. 58: 354-357.

Rieckmann KH, Campbell GH, Sax LJ, Mrema JE (1978). Drug sensitivity of Plasmodium falciparum. An *in- vitro* microtechnique. Lancet 1: 22-23.

Trape JF, Pison G, Prezioni MP, Enel C, Degrees du Lai A, Delauney V, Samb B, Lagarde E, Molez JF, Simondon F (1998). Impact of chloroquine resistance on mortality. C. R. Acad. Sci. 321: 689-697.

Wernsdorfer WH, Payne D (1991). The dynamics of drug resistance in *Plasmodium falciparum.* Pharmacol. Ther. 50: 95-121.

White NJ (1992). Antimalarial drug resistance: the pace quickens. J. Antimicrob. Chemother. 30: 571-585.

White N (1999). Antimalarial drug resistance and combination chemotherapy. Phil. Trans. R. Soc. London B. 354: 739-749.
World Health Organization (WHO) (1990). *In vitro* microtest (MARK II) for the assessment of the response of *Plasmodium falciparum* to chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine and amodiaquine. WHO: Geneva, Switzerland.
World Health Organization (WHO) (1996). World malaria situation in 1993. Weekly. Epidemiol. Rec. 71: 17-24, 25-32, 37-39, 41-48.
Yavo W, Menan EIH, Adjetey TAK, Barro-Kiki PC, Nigué L, Konan YJ, Nebavi NGF, Koné M (2002). *In vivo* sensitivity of *Plasmodium falciparum* to 4 aminoquileines and sulfadoxine-pyrimethamine in Agou (Cote d'Ivoire). Pathol. Biol. 50: 184-188.
Yavo W, Bla B, Djaman AJ, Koné M (2007). *In vitro* susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine and amino-alcohols in Abidjan (Côte d'Ivoire), Unpublished data.