

## IN VITRO SUSCEPTIBILITY OF 57 ISOLATES OF PLASMODIUM FALCIPARUM TO ATOVAQUONE AND LUMEFANTRINE IN ABIDJAN

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### ABSTRACT

To evaluate the *in vitro* chemosensitivity of *Plasmodium falciparum* isolates to lumefantrine (LUM) and atovaquone (ATO). The measurement of the *in vitro* activity of the two antimalaria molecules was conducted according to the microtest optical variant of the WHO. Out of 64 *Plasmodium falciparum* isolates tested, 57 (89%) gave interpretable results of *in vitro* culture. It was found that 67% of them were susceptible to LUM against 33% who were resisting. All were sensitive to ATO. The presence of *Plasmodium falciparum* isolates resistant to LUM could compromise the effectiveness of artemether-lumefantrine recommended in the treatment of uncomplicated malaria in Côte d'Ivoire. While the combination atovaquone / proguanil is a suitable regimen for malaria prophylaxis.

**Keywords:** *Plasmodium falciparum*, Sensibility *in vitro*, Lumefantrine, Atovaquone, Côte d'Ivoire.

### 1. INTRODUCTION

Despite a drop in the transmission and reduction of 25% in mortality compared to year 2000, malaria endemic disease remains the most common tropical parasitic disease in the world (WHO, 2011). According to the World Health Organization (WHO), malaria is the first endemic disease of which humanity is paying a heavy price. Records indicated 216 million cases of malaria in 2010, of which 81% from Africa, with 174 million cases. It was recorded more than 655,000 cases of malaria deaths in 2010, 91% in Africa alone. 86% of malaria deaths touched children under 5 years (WHO, 2011). Malaria remains the leading cause of infant mortality in the world. Most malaria deaths are caused by *Plasmodium falciparum* (Millet, 2009). For more than a decade, studies on

therapeutic effectiveness reveal increasing levels of resistance of *P. falciparum*, the main pathogen to conventional antimalarial drugs (WHO, 2009).

Malaria is a major public health problem in Côte d'Ivoire, its morbidity, mortality and the significant socioeconomic consequences. Faced with this situation, WHO recommends several strategies including early diagnosis and prompt treatment of malaria cases. However, this second aspect of malaria still faces the emergence and expansion of drug-resistant isolates of *Plasmodium falciparum*. Thus, because of the failure of monotherapy, including chloroquine and sulfadoxine-pyrimethamine, a change in strategy has become necessary in the management of malaria in most endemic countries, including Côte d'Ivoire. WHO recommends a combination of antimalarial drugs against malaria. That is why it is recommended, in Côte d'Ivoire, the use of combination therapies based on artemisinin derivatives (ACTs), namely artesunate-amodiaquine or artemether-lumefantrine for treating uncomplicated malaria (Anonyme, 2007) and the use of combination atovaquone / proguanil for prophylaxis of tourists who travel to endemic areas.

It was already reported, cases of reduced susceptibility *in vitro* of *P. falciparum* to artemisinin derivatives (Touré *et al.*, 2008; Anonyme, 2009; Bla *et al.*, 2010; Mungthin *et al.*, 2010).

To our knowledge, no study on the *in vitro* susceptibility of *P. falciparum* to LUM and ATO has been performed in our context. The emergence of *P. falciparum* resistant strains to conventional antimalarial medicines shows the need for regular monitoring of the development of resistance. So our study is to evaluate the *in vitro* sensitivity of 57 *Plasmodium falciparum* isolates from patients living in Côte d'Ivoire to atovaquone and lumefantrine.

## 2. MATERIALS AND METHODS

### 2.1. Materials

#### (a) Study Sites

This study was conducted from October 2009 to December 2010 at the Centre for Research and Fight against Malaria in the National Institute of Public Health of Côte d'Ivoire (INSP) for genomic tests. The parasitized blood sample was obtained from patients at the health center of Anonkoua-Kouté located on the outskirts of Abidjan in Abobo.

#### (b) *P. Falciparum* Isolates

The infected blood samples were obtained from patients with uncomplicated *P. falciparum* malaria in which parasite was greater than or equal to 4000 parasitized erythrocytes /  $\mu\text{l}$  of blood and who had not received any antimalarial drugs for 7 days prior consultation.

The informed consent of patients or their parents or legal guardians for children should be obtained prior to inclusion in the study.

The infected blood samples were obtained with strict aseptic conditions. Parasitized blood samples were transported in a cooler at 4°C, the same day of collection in the laboratory where

the tests *in vitro* chemo-sensitivity were performed. Parasitized red blood cells were washed three times in RPMI 1640 (Roswell Park Memorial Institute) and blood smears were stained with Giemsa and examined microscopically to determine the parasite density and confirm the *Plasmodium* species (*P. falciparum*). Samples with parasitemia ranged from 0.1% to 0.25% were used directly to test the *in vitro* susceptibility to anti malaria drugs. Those with parasite density greater than 0.25% were diluted with uninfected erythrocytes.

### (c) Antimalarial

The LUM and ATO used for testing *in vitro* susceptibility came from France (University Paris-South, Bldg 445, IBAIC and CNRS-UMR 8080, Orsay). Stock solutions of the LUM and ATO were prepared in methanol and ethanol respectively. Fold dilutions were made extemporaneously in a solution of RPMI 1640 and distributed in duplicate and triplicate in culture plates of 96 wells.

## 2.2. Methods

### (a) *In vitro* tests

The evaluation of the *in vitro* activity was performed using the optical variant of the WHO microtest (Rieckmann, 1982).

The inoculum consisted of parasitized erythrocytes, RPMI 1640 double buffered (25 mM HEPES), sodium bicarbonate 25 mM and solution of BSA (*bovine serum albumin*). For a 96-well plate, 19.2 ml of erythrocyte solution was prepared by adding 900  $\mu$ l of parasitized red blood cells (PRB), of which the hematocrit was reduced to 50% (450  $\mu$ l of PRB + 450  $\mu$ l of RPMI 1640) to 18.3 ml of RPS (1.83 ml BSA + 16.47 ml of RPMI 1640). The inoculum was distributed in the culture plate in volume of 200  $\mu$ l per well final concentrations of LUM ranges from 2.5 nM to 310 nM and that of ATO from 100nM to 0.78nM.

After brief shaking of the plate, it was placed in a candle jar (Modular incubator chamber, ICN Biomedicals, California, USA) wet and rich in CO<sub>2</sub> (5%) and incubated in a microbiological incubator (Mettler TM) at 37°C for 42 hours.

After this incubation time, three thick smears GIEMSA stained were made from pellets of blood from control wells.

The percentage of schizont maturation was determined by the ratio of the number of schizonts (asexual forms more than 2 cores) to 200 asexual counted during the microscope reading at a magnification GX100. We validated tests that their percentage of schizont maturation in control wells was at least 20%.

## (b) Statistical Analysis

Determining the values of 50% inhibitory concentrations of maturation ( $IC_{50}$ ) was made by nonlinear regression in Excel file. The threshold values used for the evaluation of *in vitro* chemosensitivity were 150 nM for LUM and 6 nM for ATO (Basco *et al.*, 1995; 1998).

## 3. RESULTS

### 3.1 In Vitro Tests

Of 64 isolates tested, 57 gave an interpretable result that is a success rate of 89%.

The geometric mean  $IC_{50}$  of ATO for all of these isolates was 1.645 nM with confidence interval (CI<sub>95</sub>) between 1.42 and 1.87 nM. The  $IC_{50}$  ranged from 3.96 nM to 0.86 nM. All isolates (100%) were susceptible to ATO (Table 1).

The geometric mean  $IC_{50}$  of LUM for all of these isolates was 23 nM (95% CI: 17.5 to 26.2 nM). The  $IC_{50}$  ranged from 3.66 nM to 184.25 nM. Ultimately, 38 of 57 isolates (67%) were susceptible to lumefantrine against 19 (33%) that were resistant with geometric mean of 8.48 nM and 168.57 nM, respectively (Table 2).

## 4. DISCUSSION

Our study on the *in vitro* susceptibility of isolates of *P. falciparum* to ATO and LUM, revealed that all isolates tested were susceptible to ATO with a geometric mean  $IC_{50}$  of 1.645 nM. These results showed that 33% of isolates were resistant to LUM with a geometric mean  $IC_{50}$  of 168.93 nM (Figure 1).

In vitro resistance to LUM had already been reported in Africa. Indeed, in a study carried out in 2009 on 115 isolates in the area of Kilifi in Kenya, 80% of isolates tested were sensitive to LUM. But 20% of the population tested was resistant to the anti-malaria (Achan *et al.*, 2009). Rates of in vitro resistance to LUM were also reported by other authors in Asia especially in Thailand where isolates of *P. falciparum* were resistant to this antimalaria (Pradines *et al.*, 2011). These results were confirmed in Tanzania (Maja *et al.*, 2013). Similarly, between 2006 and 2008, it was observed 98% and 2% for susceptible and resistant respectively to this antimalaria drug (Nsobyia *et al.*, 2010). In Senegal in 2011, various studies conducted in 165 and 93 isolates of *P. falciparum* gave isolates resistant rate of 3% and 1% respectively (Fall *et al.*, 2011; Bécaye *et al.*, 2013).

The results of all these studies carried out are in agreements with ours even if our results are higher (33% isolates resistant to LUM). These studies were conducted in malaria endemic areas where the prevalence of chloroquine resistance is very high, where Côte d'Ivoire is part of (zone 3) (Anonyme, 2012). We noted the appearance of resistance to lumefantrine and this resistance increases with time, it was 2% in Uganda in 2008 and it rose to 3% in 2011 in Senegal. This increased resistance to lumefantrine could be explained due to the high use of ACTs

including artemether-lumefantrine, sold cheaply in the black market whose antimalarial activities are not certain and taking in wrong doses, could explain the appearance of chemoresistance.

Our results were in contrast to what was obtained in 1999 and 2013 in Cameroon and Niger respectively. The results of their studies showed absence of isolates resistant to lumefantrine (Basco *et al.*, 1998; Issaka *et al.*, 2013). In any event, the presence of isolates resistant to LUM in our study may explain the occurrence of treatment failure in the artemether-lumefantrine combination reported by several authors (Touré *et al.*, 2008; Yavo *et al.*, 2010). To this must be added the case of low *in vitro* susceptibility to dihydroartemisinin, the active metabolite of artemisinin derivatives, previously reported in Côte d'Ivoire (Touré *et al.*, 2008; Bla *et al.*, 2010). This drop in sensitivity or resistance to components of ACTs could jeopardize their long-term use in the treatment of uncomplicated malaria in Côte d'Ivoire. It is therefore urgent that these drugs are prescribed rationally only to confirmed cases of malaria (WHO, 2011).

Concerning the study of *in vitro* susceptibility to atovaquone, our results are consistent with similar studies done in Africa and Asia. Indeed, isolates of *P. falciparum* from Africa were tested *in vitro* in the presence of atovaquone, results showed that all isolates were sensitive to this drug (Basco, 2003). A similar study carried out in 2006, isolates of *Plasmodium falciparum* from tourists returning from a trip to Africa, the results reported that all isolates were sensitive (Musset *et al.*, 2006). It has been observed in a similar study in areas of *P. falciparum* multiresistant in the border of Thailand in 83 isolates between 1999 and 2005, the results showed that they were sensitive to atovaquone with IC<sub>50</sub> Mean of 3.4 mM (Rommanee *et al.*, 2008). Other similar studies conducted in Guyana in 103 isolates of *P. falciparum* have confirmed our results (Anonyme, 2009).

All these studies have been done in high chloroquine resistance transmission areas (zones Saharan Africa and South-East Asia). In these areas, isolates of *P. falciparum* remain sensitive to atovaquone. Whatever the type of *P. falciparum* multidrug resistant tested atovaquone remain sensitive and this sensitivity since the use of atovaquone-proguanil in the treatment of malaria. This molecule must always be prescribed to tourists who visit areas of high malaria endemic. The lack of development of drug resistance in this molecule can be explained due to the fact that this molecule is very little or not available in African and Asian countries. It is sold in most European countries at high prices and its use is rational and reserved only to cases of malaria prophylaxis. However, in 1998 in Senegal study on the *in vitro* susceptibility of isolates of *Plasmodium falciparum* to atovaquone, revealed that isolates were resistant to this drug (Pradines *et al.*, 1998). These results are not consistent with ours but were confirmed in 2005 in a similar study of 105 isolates of *P. falciparum* in Central Africa, the results reported that 7% (4 isolates) were resistant to the anti-malarial drug with IC<sub>50</sub> > 6 mM (Menard *et al.*, 2005).

These results raise the question of threshold resistance to atovaquone that could be (IC<sub>50</sub> > 6 mM and < 1900 mM) (Musset *et al.*, 2006). On the basis of these criteria, the isolates tested in Senegal and Central Africa were sensitive because IC<sub>50</sub> found were high thus classified as

susceptible isolates. In view of these results, atovaquone-proguanil remains a suitable prescription for the prophylaxis of malaria in Côte d'Ivoire.

## 5. CONCLUSION

The existence of resistant isolates of *Plasmodium falciparum* to lumefantrine could compromise the effectiveness of artemether-lumefantrine recommended in the treatment of uncomplicated malaria in Côte d'Ivoire. Moreover, atovaquone / proguanil is a suitable therapy for malaria prophylaxis.

## 6. ACKNOWLEDGEMENTS

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## 7. DECLARATION OF CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest.

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**Table- 1.** In vitro susceptibility of isolates of P. falciparum to ATO

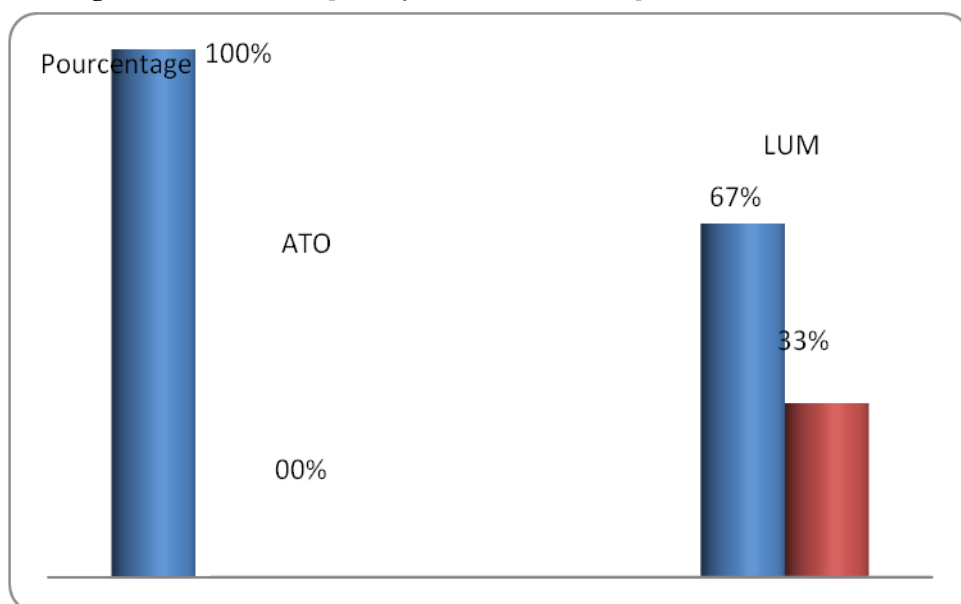
	<b>Atovaquone ( ATO)</b>
Number of tests performed.....	64
Number of tests interpretable....	57/64 (89 %)
<b><u>Sensitive Isolates ( CI<sub>50</sub> &lt; 6nM)</u></b>	
Number of isolates.....	57 (100 %)
CI <sub>50</sub> Geometrique Mean .....	1,645 nM
IC <sub>95</sub> Geometric Mean .....	1,42 – 1,87
<b><u>Resistant Isolates (CI<sub>50</sub> &gt; 6 nM)</u></b>	
Number of isolates.....	00 (00 %)
CI <sub>50</sub> Geométric Mean .....	00 nM
IC <sub>95</sub> Geométric Mean .....	00



**Table-2.** In vitro susceptibility of isolates of *P. falciparum* to LUM

	Lumefantrine (LUM)
Number of tests performed .....	64
Number of tests interpretable....	57/64 (89 %)
<u>Sensitive Isolates (<math>CI_{50} \leq 150</math> nM)</u>	
Number of isolates .....	38 (67 %)
$CI_{50}$ Geométric Mean .....	8,48 nM
$IC_{95}$ Geométric Mean.....	6,57 – 10,28
<u>Résistant Isolates (<math>CI_{50} &gt; 150</math> nM)</u>	
Number of isolates.....	19 (33 %)
$CI_{50}$ Geométric Mean .....	168,93 nM
$IC_{95}$ Geométric Mean .....	123,20 – 191,37

**Figure- 1.** In vitro susceptibility of *Plasmodium falciparum* to ATO and LUM



Iso S= isolates sensitive

Iso R = isolates resistant

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