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MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XVII. BASELINE MONITORING OF ATOVAQUONE-RESISTANT *PLASMODIUM FALCIPARUM* BY *IN VITRO* DRUG ASSAYS AND CYTOCHROME *B* GENE SEQUENCE ANALYSIS

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Abstract. Atovaquone is a new broad-spectrum antiprotozoal drug with high *in vitro* activity against multidrug-resistant *Plasmodium falciparum*. Its specific action against protozoans is based on the inhibition of the parasite cytochrome *bc1* complex of the mitochondrial electron transport system. Protozoans may develop atovaquone resistance by the selection of a mutant cytochrome *b* gene. With the increasing availability of atovaquone-proguanil combination for prophylaxis and treatment of malarial infections, it is necessary to establish baseline data on atovaquone sensitivity before the drug is introduced massively in an endemic region. For this purpose, the activity of atovaquone was assessed indirectly by *in vitro* drug sensitivity assays with several serum substitutes and DNA sequencing of the cytochrome *b* gene. Using the standard *in vitro* assay procedures with 10% human serum, the geometric mean 50% inhibitory concentration (IC_{50}) for atovaquone was calculated to be 1.15 nM (range = 0.460–4.17 nM), while the use of 10% fetal calf serum resulted in lower IC_{50}s (geometric mean = 0.575, range = 0.266–2.20 nM). The use of Albumax, a lipid-enriched bovine albumin, over the same concentration range (0.25–16 nM) showed poor results. None of the 37 isolates with an atovaquone $IC_{50} < 4.17$ nM displayed any mutation. Further monitoring of atovaquone-resistant *P. falciparum* is warranted for the rational use of this new antimalarial drug.

INTRODUCTION

Atovaquone, a naphthoquinone derivative, is a new antimalarial drug with high activity against multidrug-resistant *Plasmodium falciparum*, as well as against other protozoans.[1] It is one of the rare compounds belonging to an entirely new chemical class that attained clinical phase of development. *In vitro* studies on clinical isolates of *P. falciparum* from different geographic origins have shown its high activity.[2,3] Preliminary clinical studies on atovaquone administered as monotherapy have shown its rapid action to clear parasitemia,[4,5] but a high recrudescence rate of 33% was observed in a study in Thailand.[5] These recrudescent parasites were highly resistant *in vitro* to atovaquone, with an approximately 1,000-fold increase in inhibitory concentrations. Additional *in vitro* studies have identified doxycycline and proguanil (biguanide), but not its non-biguanide metabolite cycloguanil or pyrimethamine (non-biguanide dihydrofolate reductase inhibitor), as synergistic partners of atovaquone.[6,7] Clinical studies confirmed the *in vitro* results with high cure rates for atovaquone-proguanil and atovaquone-doxycycline combinations, but not atovaquone-pyrimethamine.[5] Further clinical studies on the atovaquone-proguanil combination conducted in different parts of the world have largely confirmed its efficacy, tolerance, and safety for both treatment and prophylaxis.[8–16] At the present time, the atovaquone-proguanil combination is available in many non-endemic countries for prophylaxis of travelers visiting endemic areas for up to one month, and in some endemic countries, the drug combination was available until recently for the treatment of multidrug-resistant *P. falciparum* infections through the drug donation program.[17]

The mechanism of action of atovaquone is based on the selective inhibition of the cytochrome *bc1* complex of the mitochondrial electron transport system in malaria parasites.[18–20] Proguanil itself does not have any effect on mitochondrial functions, and its weak inhibitory action (nor the strong inhibitory action of cycloguanil and pyrimethamine) on dihydrofolate reductase does not seem to be involved in the synergistic interaction with atovaquone. One recent study has suggested that proguanil may enhance the ability of atovaquone to perturb mitochondrial membrane potential at lower doses, but the exact mechanism of synergy between these two drugs is not known.[7] Atovaquone resistance has been associated with the capacity of malaria parasites to maintain normal functions of the mitochondrial electron transport despite the presence of high drug concentrations.[21] The underlying genetic basis for atovaquone resistance has been linked to the presence of specific mutations in the cytochrome *b* gene in both rodent malaria parasites and laboratory-adapted strains of *P. falciparum*.[21–23]

In the African continent, up to one million doses of the atovaquone-proguanil combination had been available annually for the treatment of uncomplicated *P. falciparum* infections in some countries through the drug donation program in recent years.[17,24] Although Cameroon was not one of the beneficiary countries until the present time, the massive introduction of the drug combination and its expected uncontrolled distribution in Africa would require close monitoring of drug efficacy. With this perspective in mind, the present study was undertaken to establish the baseline level of *in vitro* atovaquone activity against Cameroonian clinical isolates, analyze the cytochrome *b* gene sequence, and assess the potential of fresh clinical isolates to develop atovaquone resistance.

MATERIALS AND METHODS

Parasites. Venous blood samples were obtained after informed consent was obtained from children ≥12 years old and adults spontaneously consulting the Nlongkak Catholic Missionary Dispensary in Yaounde, Cameroon in 2001–2002 if the following criteria were met: signs and symptoms of acute uncomplicated malaria, the presence of *P. falciparum* at a parasitemia ≥0.1% without other *Plasmodium* species, and a negative Saker-Solomons urine test result for 4-aminoquinolines.[25] Young children (<12 years old), pregnant women, anemic patients (hematocrit <20%), and patients with signs and symptoms of severe and complicated malaria

were excluded. The patients were treated with oral amodiaquine, the first-line drug in Cameroon, and followed-up by the dispensary staff to ensure parasite and fever clearance on or before day 4. This study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

***In vitro* drug sensitivity assays.** Atovaquone hydrochloride was kindly provided by GlaxoWellcome (Stevenage, Hertfordshire, United Kingdom). A stock solution and two-fold dilutions were prepared in methanol, and 96-well culture plates were pre-coated with dilutions (final concentrations ranging from 0.25 nM to 16 nM) in triplicate and air-dried.

Blood samples were washed three times in RPMI 1640 culture medium within two hours after blood extraction. Infected erythrocytes were suspended in the RPMI 1640 medium containing 25 mM HEPES, 25 mM $NaHCO_3$, and serum or serum substitute at a hematocrit of 1.5%. One of the following sera or serum substitute was added to supplement the culture medium: 10% non-immune human sera pooled from four European donors, 10% fetal calf serum (tested for mycoplasma) from two different suppliers (batch no. 5-41201; Integro b. v., Amsterdam, The Netherlands and Seromed®, batch 8R02; Biochrom KG, Berlin, Germany) or 0.5% Albumax II (Invitrogen Life Technologies, Cergy Pontoise, France). The initial parasitemia was adjusted to 0.6% by adding fresh uninfected erythrocytes if the parasitemia was > 1%. The *in vitro* isotopic microtest was performed as previously described.[26] The 50% inhibitory concentration (IC_{50}), defined as the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthine measured in the drug-free control wells, was determined by a non-linear regression analysis using the Prism™ software (GraphPad Software, Inc., San Diego, CA).

Polymerase chain reaction and sequencing of DNA. Parasite DNA was extracted from a red blood cell pellet as previously described.[26] The *P. falciparum* mitochondrial cytochrome *b* gene was amplified by a nested polymerase chain reaction. The synthetic oligonucleotides were designed from the complete mitochondrial DNA sequence (GenBank accession number M99416).[19] In the primary reaction, a 1.1-kilobase pair fragment spanning almost the entire coding region of the cytochrome *b* domain was amplified using the primer pairs PFCYTB-1, 5′-TTAGTTAAAGCACACTTAATAAATTACCC-3′ (forward primer, nucleotides 22–50; nucleotide numbering based on cytochrome *b* domain, the start codon designated as nucleotide 1 in this study corresponds to nucleotide 4758 in M99416) and PFCYTB-2R, 5′-GCTTGGGAGCTGTAATCATAATGTGTTCG-3′ (reverse primer, nucleotides 1121–1093). The reaction mixture consisted of genomic DNA, 15 picomole of each primer, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM $MgCl_2$, 200 μM of deoxynucleoside triphosphates (mixture of dGTP, dATP, dTTP, and dCTP), and one unit of *Taq* DNA polymerase (Roche Diagnostics, Meylan, France) in a total volume of 50 μL. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for two minutes for the first cycle and 30 seconds in subsequent cycles, 50°C for one minute for the first cycle and 30 seconds in subsequent cycles, and 72°C for one minute in all cycles, for a total of 30 cycles.

A secondary amplification was performed on the primary amplification product with internal primers PFCYTB-3 (forward primer, 5′-ATTTATGATATTTATTGTAACTGC-3′, nucleotides 342–365) and PFCYTB-4R (reverse primer, 5′-AGTTGTTAAACTTCTTTGTTCTGC-3′, nucleotides 906–883). Except for the primers and DNA template, the reaction mixture and thermal cycler program for the secondary amplification reaction were identical to that of the primary reaction.

The 565-basepair product was purified using the High Pure PCR Purification kit (Roche Diagnostics). The amplification product was marked with fluorescent nucleotides following the manufacturer's instructions (Perkin Elmer Corp., Les Ulis, France). The ABI Prism automated DNA sequencer (Perkin Elmer Corp.) was used to sequence the extension product.

Data interpretation. Parasite growth in drug-free control wells containing RPMI 1640 medium supplemented with different sera or serum substitute in triplicate was expressed as the relative growth index, defined as the percentage of counts per minute (cpm) obtained with Albumax or fetal calf serum, as compared with cpm obtained with 10% human serum. Wild-type and mutant cytochrome *b* alleles were defined on the basis of amino acid sequence differences between TM93-C1088 (a recrudescent isolate obtained from a Thai patient after treatment with atovaquone-pyrimethamine) and other *P. falciparum* clones that have been selected during *in vitro* culture by stepwise exposure to increasing concentration of atovaquone.[23] These parasites display the following amino acid substitutions, singly or in combination: Met133Ile, Tyr268Ser, Lys272Arg, Pro275Thr, Gly280Asp, Ile283Met, and Val284Lys. The DNA sequence of cytochrome *b* gene was compared with the IC_{50} of atovaquone.

RESULTS

The *in vitro* activity of atovaquone was determined in 37 isolates. Among different sera or serum substitute used in this study, 10% fetal calf serum yielded a satisfactory parasite growth in drug-free control wells and consistent parasite growth inhibition at 16 nM, i.e., ≥10-fold difference in the incorporation of tritium-labeled hypoxanthine between drug-free control and the highest drug concentration, allowing an accurate plot of a sigmoid curve by the non-linear regression model. Using 10% fetal calf serum, the geometric mean IC_{50} for atovaquone was calculated to be 0.575 nM (n = 37, range = 0.266–2.20 nM) (Figure 1). Due to the wide variation in quality and components of animal sera, a second batch of fetal calf serum obtained from another supplier was tested in parallel with the first batch of fetal bovine serum for six isolates. Although the second batch resulted in a better parasite growth in drug-free control wells, with an average growth index of 1.9, similar regression curves and IC_{50} values were obtained with both batches of fetal calf serum (Table 1).

Using 10% non-immune human serum, interpretable results were obtained for 33 of the 37 isolates. Four assays with human serum were uninterpretable due to inadequate parasite inhibition at 16 nM and/or relatively low hypoxanthine incorporation in the control wells, compared with that obtained at 16 nM. On the average, the atovaquone IC_{50} was 2.0 times higher with 10% human serum (geometric mean = 1.15 nM, range = 0.460–4.17 nM) than with 10% fetal bovine serum. Although parasite growth varied widely with the two

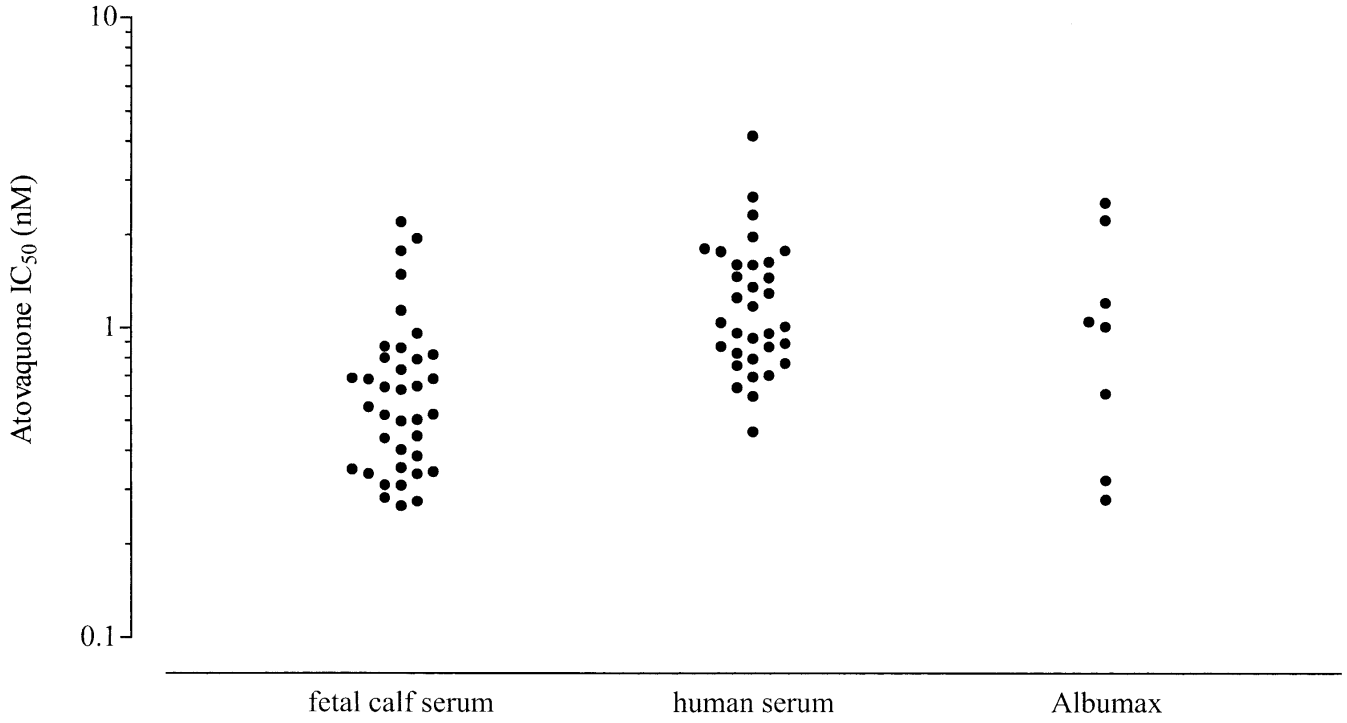

FIGURE 1. Distribution of the atovaquone 50% inhibitory concentration (IC_{50}) for isolates of *Plasmodium falciparum* in relation to serum supplement. While all 37 isolates had interpretable results with 10% fetal calf serum, 4 of 37 isolates and 8 of 16 isolates had uninterpretable results with 10% human serum and 0.5% Albumax, respectively. The poor results with Albumax led us to abandon further experiments with this serum substitute.

serum supplements (range of growth index of fetal bovine serum versus human serum = 0.40–2.6) in individual isolates, the mean growth index with fetal bovine serum, compared with that of human serum, was 1.1.

Albumax (0.5%) was used as a serum substitute to determine the atovaquone IC_{50} for 16 isolates. The growth index of 2.3 was satisfactory, but 8 of 16 assays were uninterpretable due to either non-sigmoidal distribution of experimental points or, more frequently, inadequate parasite growth inhibition at 16 nM. Of eight interpretable assays, only three had similar IC_{50}s as those obtained with fetal calf serum, while the others were between two- and eight-fold higher than the corresponding IC_{50}s obtained with fetal calf serum. Because of these inconsistent results, the use of Albumax as a serum substitute was abandoned after the first 16 assays.

The DNA sequence of cytochrome *b* was available for all 37 isolates. All sequences were identical at the nucleotide level, with no mutation within the 565-basepair fragment amplified by the polymerase chain reaction, which includes all codons currently known to undergo mutation in *P. falciparum*. The wild-type cytochrome *b* gene sequences and low atovaquone IC_{50}s (<4.2 nM using 10% human serum) indicate that all isolates were sensitive to atovaquone.

TABLE 1

Comparison of fetal calf sera from two different sources for parasite growth and determination of atovaquone IC_{50}*

	Fetal calf serum 1		Fetal calf serum 2	
Isolate	cpm†	IC_{50} (nM)	cpm†	IC_{50} (nM)
80/01	9,710 ± 898	0.725	4,860 ± 204	0.791
81/01	14,300 ± 1,150	0.579	9,080 ± 190	0.871
82/01	19,210 ± 1,120	1.83	7,230 ± 264	1.78
84/01	7,660 ± 296	0.295	5,080 ± 405	0.282
85/01	9,230 ± 124	0.419	3,905 ± 289	0.337
86/01	4,850 ± 532	0.502	3,640 ± 242	0.446

* IC_{50} = 50% inhibitory concentration.
† Incorporation of ^{3}H-hypoxanthine in drug-free control wells. Results are expressed as the mean ± SD counts per minute (cpm) of three wells.

DISCUSSION

Previous *in vitro* studies have shown the high activity of atovaquone against *P. falciparum* isolates originating from various African countries and imported into France by returning travelers.[2,3] Atovaquone IC_{50}s of Cameroonian isolates determined in the present study are within the range of IC_{50}s of isolates originating from various African countries. We have assessed the utility of other serum substitutes for *in vitro* drug sensitivity assays due to the difficulties in obtaining human sera from non-immune donors. Fetal calf serum seemed to be a suitable substitute, yielding consistent parasite growth and interpretable *in vitro* assay results for atovaquone. The mean parasite growth was comparable with both sera, and atovaquone IC_{50} was 2.0 times higher with human serum. Although the reasons for lower atovaquone IC_{50} with fetal bovine serum were not investigated, it may be conjectured that the differences in the plasma protein composition may favor drug entry into parasites either by the availability of more unbound form of atovaquone or by carrier-mediated processes. In contrast, the use of Albumax resulted in considerably higher atovaquone IC_{50}s, and the concentration range used in this study was probably too low to obtain complete inhibition of parasite growth.

Previous studies have shown that atovaquone-resistant laboratory-adapted parasites can be selected by continuous *in vitro* culture under drug pressure with 10 nM of atovaquone.[23,27–29] Once acquired, mutations seem to be stable in mutant parasites cultured subsequently without drug pressure. Alignment of cytochrome *b* sequences from different organisms and molecular modeling have suggested that only single or double substitutions of amino acid residues at or near the atovaquone binding site within the enzyme may be required to develop atovaquone resistance in protozoans.[23] In *P. falciparum*, based on the limited data available so far, a single Tyr268Ser or Tyr268Asn substitution was associated with clinical failure to atovaquone treatment.[23,30–32] Other mutations may occur by selection in laboratory-adapted parasites. However, in field isolates from areas where atovaquone has not been introduced, such as in India and Cameroon, the mutations associated with atovaquone resistance have not been observed, and natural parasite populations seem to maintain the highly conserved cytochrome *b* gene sequence.[33,34]

Our results show the high *in vitro* activity of atovaquone against Cameroonian isolates and are consistent with those of previous studies on fresh isolates from various African countries.[2,3] None of the isolates displayed any evidence for atovaquone resistance. Atovaquone IC_{50}s were within a low nanomolar range. Furthermore, the cytochrome *b* gene sequence was highly conserved, with no mutation among 37 isolates. These data, taken together, suggest that at present naturally occurring atovaquone-resistant *P. falciparum* is probably absent in Yaounde, Cameroon. Further monitoring of atovaquone resistance would be necessary should the drug be introduced in central Africa for either the treatment of *P. falciparum* malaria or prophylaxis/treatment of other protozoan diseases.

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