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Original article

Clinical atovaquone-proguanil resistance of *Plasmodium falciparum* associated with cytochrome *b* codon 268 mutations

Lise Musset [a,b,*], Olivier Bouchaud [c], Sophie Matheron [d], Laurent Massias [e], Jacques Le Bras [a,b]

[a] *Laboratoire de biologie animale et parasitaire, EA209, Université Paris Descartes, 4 av de l'observatoire, 75006 Paris, France*
[b] *Centre National de Référence Paludisme, APHP, Hôpital Bichat-Claude Bernard, 46 rue Henri Huchard, 75877 Paris Cedex 18, France*
[c] *Unité de maladies infectieuses et tropicales, APHP, Hôpital Avicenne, 125 rue de Stalingrad, 93000 Bobigny, France*
[d] *Unité de maladies infectieuses et tropicales, APHP, Hôpital Bichat-Claude Bernard, 46 rue Henri Huchard, 75877 Paris Cedex 18, France*
[e] *Laboratoire de toxicologie, APHP, Hôpital Bichat-Claude Bernard, 46 rue Henri Huchard, 75877 Paris Cedex 18, France*

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Abstract

Plasmodium falciparum resistance to atovaquone-proguanil has so far been associated with Y268S or Y268N mutations in cytochrome *b*, although these changes were identified in only seven of the 11 treatment failures. Here, we describe 10 new cases of atovaquone-proguanil treatment failures among which the parasite resistance was confirmed in six cases, either by identifying correct plasma drug concentrations or by observing in vitro atovaquone resistance. Resistance was consistently associated with codon 268 mutations (Y268S or a previously unidentified mutation, Y268C). Notably, mutations were not detected before the treatment but only after the drug exposure.

Keywords: *Plasmodium falciparum*; Chemotherapy; Resistance; Cytochrome *b*; Atovaquone; Proguanil; Malarone

1. Introduction

In 1996, the atovaquone-proguanil (AP) combination was registered in North America and Europe for the prophylaxis and treatment of malaria. While this safe and efficient combination is increasingly used in developed countries, its high cost precludes a large use in endemic countries. Atovaquone, a ubiquinone analogue binding to cytochrome *b* of plasmodial mitochondria, inhibits electron transfer of the respiratory chain [1]. Proguanil is mainly considered as a pro-drug of cycloguanil, an inhibitor of the dihydrofolate reductase also involved in pyrimidine biosynthesis. However, in combination with atovaquone, proguanil, by itself, lowers the effective concentration at which the former collapses the mitochondrial membrane potential [2]. The proguanil target and the detailed mechanism implicated in AP synergy remain unknown. Since the introduction of AP combination, 11 cases of treatment failures have been published in travelers returning from Africa [3–7]. Seven failures exhibited a modification of codon 268 of the cytochrome *b* gene (*pfcytb*268), mostly from tyrosine (Y) to serine (S) and four failures were reported without any *pfcytb* mutation. Thus, the usefulness of *pfcytb*268 mutations for predicting *Plasmodium falciparum* AP resistance has been questioned [8]. The limitation for understanding the failure mechanisms may also have been related to an insufficient description of the cases, impairing discrimination between parasite resistance and poor drug absorption. Atovaquone is a very

Abbreviations: AP, atovaquone-proguanil; *pfcytb*, cytochrome *b* gene; *pfdhfr*, dihydrofolate reductase gene; IC_{50}, inhibitory concentration 50%; C, cysteine; *pfmsp*, merozoite surface protein gene; N, asparagine; PCR, polymerase chain reaction; S, serine; Y, tyrosine; WHO, World Health Organization.

* Corresponding author. Laboratoire de Parasitologie-Mycologie, Hôpital Bichat-Claude Bernard, 46 rue Henri Huchard, 75877 Paris Cedex 18, France. Tel.: +33 140 257 899; fax: +33 140 256 763.
E-mail address: lisemusset@gmail.com (L. Musset).

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lipophilic drug and its low bioavailability is 4-fold increased (reaching 23%) with simultaneous intake of fatty food. This poor absorption of atovaquone may induce treatment failures without parasite resistance. With this in mind, this study was designed to investigate putative causes of 10 additional AP treatment failures identified over a 3-year period.

2. Materials and methods

2.1. Patients

Between January 2003 and September 2005, six cases of AP treatment failures were identified among 298 patients suffering from uncomplicated *P. falciparum* malaria and treated with AP (four tablets daily for 3 days). Most patients, being 9–75 years of age, had returned from Central or West African countries. Eighty percent were living in France but were native from the country of infection. In our hospital, a voluntary monitoring of malaria treatment efficacy is systematically proposed. Based on the WHO standard protocol, follow-up plans clinical and parasitological examinations between Day 0 (i.e. first day of treatment) and Day 28 (generally, Day 3, Day 7 and Day 28). Early and late treatment failures were defined following WHO criteria [9]. Informed consent was not required for this study as the following procedures are part of the French national recommendations for the care and surveillance of malaria [10]. As our laboratory is the national reference centre for malaria, we were informed of four additional cases of late AP treatment failure. In agreement with national consensus, all patients with failure were re-treated with quinine.

2.2. Investigation of treatment failures

For all treatment failure patients, atovaquone in vitro susceptibility testing, *pfcytb* and *pfdhfr* genotypings, and merozoite surface proteins 1 and 2 (*pfmsp*1 and *pfmsp*2) polymorphism analyses were performed on Day-0 and Day-of-failure isolates. Measurements of atovaquone, proguanil and cycloguanil concentrations were determined from plasma to assess for correct drug absorption and compliance.

2.2.1. Atovaquone in vitro susceptibility testing

An in vitro isotopic test was used to determine the atovaquone inhibitory concentration 50% (IC_{50}). The in vitro atovaquone resistance threshold is between 40 and 1900 nM [11].

2.2.2. Cytochrome b genotyping

The entire *pfcytb* gene was analysed by sequencing [11]. Position of *pfcytb*268 was further investigated using a nested polymerase chain reaction followed by a restriction full-length polymorphism method.

2.2.3. Pfdhfr genotyping

The three major *pfdhfr* mutations (at positions 51, 59, and 108) associated with cycloguanil resistance were studied with a restriction method.

2.2.4. Parasite population analysis

Parasite diversity within isolates was determined by a fragment-analysis method of *pfmsp*1 and *pfmsp*2 polymorphisms. This method allows for the detection of all genotypes accounting for more than 2% of the whole parasite population [12].

2.2.5. Drug measurements

Determinations of drug concentrations in plasma were performed by reverse phase high performance liquid chromatography. The lower limits of quantification were 1.4 μM, 0.03 μM and 0.04 μM for atovaquone, proguanil and cycloguanil, respectively. Results were compared to effective atovaquone plasma concentrations in malaria treatment: between 3 and 20 μM, 0.6 and 18 μM, 0.3 and 2.2 μM on Day 3, Day 8 and Day 21, respectively [13].

2.3. Classification of treatment failures

To define a reliable allele of AP resistance, treatment failures were classified into three categories:

(i) *Failures in absence of AP resistance*: incorrect plasma drug dosages associated with Day-of-failure parasites in vitro susceptible to atovaquone,
(ii) *Failures caused by AP resistance*: correct drug dosages or Day-of-failure parasites resistant to atovaquone, and
(iii) *Indeterminate*.

3. Results

Of the 10 analysed atovaquone-proguanil treatment failures, parasite resistance was present, absent and indeterminate in five, three and two cases, respectively (Table 1). In absence of travel between Day 0 and Day-of-failure, reinfection was excluded in all the patients.

In patients 1, 2 and 3, the atovaquone in vitro phenotype excluded parasite resistance, with susceptible values below the in vitro minimum resistance threshold of 40 nM. Day 3 drug level measurements confirmed poor absorption or bad compliance. In these cases, no *pfcytb* polymorphism was identified in parasites isolated before and after the treatment failure.

In patients 4–8, drug level measurements or the atovaquone in vitro phenotype confirmed parasite resistance with susceptible values far above the resistance threshold. All these cases were associated with triple mutant *pfdhfr* genotype and carried a *pfcytb*268 mutation, these being Y268S ($n = 3$) or a novel mutation Y268C ($n = 2$). In addition, a change S299N was observed in Day-0 and Day-of-failure parasites from patient 4. Pre-treatment isolates were available for all cases except Day-0 sample for patient 5. They showed the parasites to be *pfcytb* wild-type on Day 0, and until Day 7 for patient 4 and Day 10 for patient 8.