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## Different mutation patterns of atovaquone resistance to *Plasmodium falciparum* *in vitro* and *in vivo*: rapid detection of codon 268 polymorphisms in the cytochrome b as potential *in vivo* resistance marker

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### Abstract

**Background:** Resistance of *Plasmodium falciparum* to atovaquone *in vitro* and *in vivo* has been associated to mutations in the parasite cytochrome b gene.

**Methods:** Cultures were sequentially subjected to increasing doses of atovaquone alone or in combination with cycloguanil and the cytochrome b gene was sequenced. Additionally, we investigated the parasite cytochrome b gene of a patient returning from Mali with Malarone<sup>®</sup> treatment failure *in vivo*.

**Results:** All strains that survived atovaquone concentrations *in vitro* of  $2 \times 10^{-8}$  to  $2 \times 10^7$  M showed the M133I mutation and one strain with the highest atovaquone concentration the additional mutation L171F. Sequencing of the *in vivo* treatment failure revealed a point mutation at codon 268 resulting in an amino acid change from tyrosine to serine. Based on the repeated emergence of mutations at codon 268, but no detection of alterations at codon 133 *in vivo*, we developed a detection method for the diagnostic of codon 268 polymorphisms as a potential atovaquone/proguanil resistance marker. A nested PCR with 3 different pairs of primers for the second round was designed. Each product was digested with restriction enzymes, capable to distinguish the wild type from the two reported mutations at codon 268.

**Conclusion:** Mutations at codon 268 of the parasite cytochrome bc<sub>1</sub> gene are associated with atovaquone/proguanil treatment failure *in vivo* and can be used as potential resistance marker. This method provides a novel and robust tool to investigate the relevance of codon 268 polymorphisms as resistance marker and to monitor the further emergence of atovaquone/proguanil resistance.

### Background

The rapid emergence of resistance to standard antimalarial drugs has become a serious global health problem in endemic countries. For affected travellers returning to in-

dustrialised countries, effective treatment is available and resistance is as yet not a frequent problem in the treatment of falciparum malaria. The recently introduced drug Malarone<sup>®</sup> is a combination of atovaquone and proguanil

and is used for treatment and prophylaxis. There is certain evidence that parasites may quickly develop resistance to atovaquone and proguanil. When treated with atovaquone alone, one study showed that 33% of patients experienced recrudescence of parasitaemia [1]. In combination with proguanil, cure rates from 99–100% were achieved [2–7]. In 2002, the first case of *in vivo* resistance to atovaquone and proguanil in a non-immune European traveller, returning from Nigeria, was reported [8].

Atovaquone acts by inhibiting mitochondrial electron transport [9] and collapsing mitochondrial membrane potential [10]. It has been suggested that atovaquone, based on its structural similarity to ubiquinol, binds to the parasitic cytochrome  $bc_1$  [11]. Mutations in the cytochrome  $bc_1$  gene of the parasite mitochondrial genome have been described as conferring atovaquone resistance. Two mutations in *Pneumocystis carinii* at the ubiquinol-binding pocket ( $Q_0$  domain) are associated with atovaquone prophylaxis failure [12]. In *in vitro* resistant *Toxoplasma gondii* lines two mutations at codon 129 and 254 were found to confer atovaquone resistance. Atovaquone-resistant *Plasmodium yoelii* lines have been derived by sub-therapeutic treatment of infected mice. Five mutations near the putative atovaquone binding pocket have been identified, including a substitution of tyrosine by cysteine at codon 268 [13]. In a similar study three mutations at the cytochrome b gene of atovaquone resistant *Plasmodium berghei* lines were found to be associated with resistance to atovaquone. The mutations at codon 133 or 144, in addition to an amino acid change at codon 284, led to increased resistance levels.[14]. Atovaquone resistant lines of *Plasmodium falciparum* have been derived *in vitro* by surviving various concentrations [15]. An initial mutation at codon 133 was found to confer low resistance, which could be increased by an additional mutation in the domain from codon 272 to codon 280.

*In vivo* the cytochrome  $bc_1$  sequence of a *P. falciparum* isolate from a Thai patient with recrudescence after atovaquone and pyrimethamine treatment showed a mutation at codon 268 resulting in the substitution of tyrosine by serine [1,15]. An amino acid change to asparagine at the same codon was described in an English patient travelling to Nigeria who failed atovaquone/proguanil therapy [8].

In this report we describe laboratory derived mutations of the cytochrome  $bc_1$  gene of *P. falciparum* after sub-curative administration of atovaquone alone or in combination with cycloguanil. These *in vitro* changes have been compared with mutations of an *in vivo* isolate derived from a patient with recrudescence after atovaquone/proguanil treatment. Based on this information we developed a nov-

el PCR-RFLP method for the detection of mutations at codon 268, associated with resistance to atovaquone/proguanil.

## Material and Methods

### **In vitro induction of atovaquone resistance**

The *P. falciparum* laboratory line K1 [16] was cultivated *in vitro* according to Trager & Jensen [17] with 5% haematocrit and medium containing RPMI, glucose, gentamycin sulfat, hepes, sodium bicarbonate, hypoxanthine (all purchased from SIGMA-ALDRICH, Taufkirchen, Germany) and human sera. Fresh erythrocytes and plasma bottles were purchased from the Red Cross. Cultures were split every 2 days according to cell growth. The selection of drug resistance followed a modified protocol of Korsinczky, 2000 [15]. Parasites were first cultured in one flask and split into eight cultures when parasitaemia reached approximately 2%. The eight cultures were maintained until the parasitaemia reached approximately 5%. Parasitised red blood cells from one of the eight flasks were cryopreserved as the atovaquone-sensitive parent. From the remaining flasks, one was cultivated as control without drugs, 3 were cultivated with medium containing  $10^{-8}$  M atovaquone and 3 flasks with  $10^{-8}$  M atovaquone plus 0.88 ng/ml cycloguanil. Cycloguanil concentration was calculated as atovaquone/proguanil ratio of 2.5 in Malarone® tablets and 30% metabolism of proguanil to cycloguanil, achieving an atovaquone/cycloguanil ratio (weight) of 8.33. The parasites that survived were sequentially subjected to  $2 \times 10^{-8}$ ,  $5 \times 10^{-8}$ ,  $10^{-7}$  and  $2 \times 10^{-7}$  M atovaquone or the combination of atovaquone and cycloguanil, respective. Aliquots of parasites were removed after surviving each drug concentration for cryopreservation and DNA preparation.

### **In vivo resistance**

The blood sample TN352 of a *P. falciparum* case presented after self treatment with a complete course of Malarone® was collected within the network TropNetEurop. The sample NGATV01 of a Malarone® treatment failure was kindly provided by Dr. Warhurst, London School of Hygiene and Tropical Medicine/UK [8].

### **Sequencing of Plasmodium falciparum cytochrome b gene**

Parasite DNA was extracted by use of BIO RAD InstaGene Matrix. A fragment of the cytochrome  $bc_1$  gene was amplified as described by Korsinczky [15], using primers CYTb1 and CYTb2. PCR was carried out using 2.5 U Qiagen Hotstar taq, 1.5 mM  $Mg^{2+}$ , 1  $\mu$ M of each primer and 0.2 mM dNTP's. The reaction mixture was initially heated at 93°C for 15 min and then cycled at 93°C for 50s, 45°C for 50s and 72°C for 1 min over 40 cycles. PCR products were purified from the gel using Qiaex(II) Kit (Qiagen) and ligated into the pCR®2.1 Topo vector (Invitrogen TA-TOPO

**Table 1: Method for detecting codon 268 mutations: primer sequences for outer and nested PCR (5' -> 3')**

Primer Name	Sequence
CYTb1	CTCTATTAATTTAGTTAAAGCACA
CYTb2	ACAGAATAATCTCTAGCACC
CYTb3	AGCAGTAATTTGGATATGTGGAGG
CYTb5	GGTTTACTTGGAACAGTTTTTAACAaTG *
CYTb6	TGAATGGTACTTTCTACCAgTT *
CYTb7	ACCTGAATGGTACTTTCTACaATaT *

\* Small letters indicate base changes to provide digest sites for restriction enzymes to distinguish wild type from mutations at codon 268

**Table 2: Method for detecting codon 268 mutations: primer pairs for amplification with size of amplification product and restriction enzyme for following digest**

Codon 268	Type	Primer pair	Product size	Cutting enzyme	Fragment length
<b>TAT</b>	Outer PCR	CYTb1 + CYTb2	939 bp		
	Wild type	CYTb3 + CYTb5	384 bp	Nsil	359 + 25
<b>TCT</b>	Y268S	CYTb2 + CYTb6	171 bp	AlwNI	147 + 24
<b>AAT</b>	Y268N	CYTb2 + CYTb7	174 bp	Sspl	150 + 24

Nsil cuts TAT; AlwNI cuts TCT; Sspl cuts TAT and TCT, but not AAT

Cloning kit). *Escherichia coli* TOP10 were subsequently transformed with the ligation mix and positive clones selected and propagated. The plasmids were prepared using QIAprep Spin Plasmid Kit, then sequenced in both directions using ABI Prism Big dye Terminator kit employing the vector primers M13 forward and M13 reverse and the internal primers CYTb3 and CYTb4, with a 3 times coverage of the gene. [15]. Sequencing was done on ABI Prism 377 (Perkin-Elmer), proof-reading and translation was done using Seqman software from DNASTar, or was outsourced to MWG Biotech (MWG-Biotech Ebersberg, Germany).

#### PCR-RLFP method for codon 268 mutations

A nested PCR was designed using CYTb1 and CYTb2 as outer primers and 3 different pairs of nested primers to distinguish the 3 known polymorphisms at codon 268. Primer sequences are shown in table 1. PCR product sizes are listed in table 2. For the primary amplification reaction a mix containing 0.125  $\mu$ M of each outer primer, 0.2 mM dNTP's, 1.5 mM  $Mg^{2+}$  and 0.5 U Taq polymerase (Qbiogene, Carsbad, Canada) was initially heated at 94°C for 5 min and then cycled at 94°C for 50s, 50°C for 50s and 70°C for 1 min over 35 cycles with a final extension at 70°C for 5 min. For the secondary amplification, 1  $\mu$ l of PCR product was added to the mastermix containing 0.5  $\mu$ M primers and dNTP,  $MgCl_2$ , Taq Polymerase as above. PCR conditions were 94°C for 5 min, 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s and a final extension at 72°C for 5 min for the primer pairs CYTb3/

CYTb5 as well as for CYTb2/CYTb6. CYTb2/CYTb7 were annealed at 45°C. The products of the second round were confirmed by electrophoresis in ethidium bromide-stained agarose gel.

For RFLP analysis 5  $\mu$ l of PCR product was mixed with 1 U of the appropriate enzyme (see Table 2) and its specific buffer in a total volume of 22  $\mu$ l and incubated at 37°C over night. The result was detected by electrophoresis in ethidium bromide-stained agarose gel.

## Results

### **In vitro induction of resistance against atovaquone alone or the combination of atovaquone and cycloquanil in a *P. falciparum* KI strain results in mutations in the cytochrome bc<sub>1</sub> gene**

All parasites that survived an atovaquone concentration of  $2 \times 10^{-8}$  M,  $1 \times 10^{-7}$  M and  $2 \times 10^{-7}$  M in the medium showed a single base mutation at position 399 in the cytochrome bc<sub>1</sub> gene. The mutation resulted in an amino acid change from methionine (ATG) to isoleucine (ATT) at codon 133 (M133I). The same mutation was seen in parasites that were cultured in a medium containing the same concentration of atovaquone in combination with cycloquanil. The parasite line AT200 survived  $2 \times 10^{-7}$  M atovaquone in the medium. Sequencing of the cytochrome bc<sub>1</sub> gene identified a additional mutation at position 813 resulting in an amino acid change at codon 271 from leucine (TTA) to phenylalanine (TTC).

### **The *P. falciparum* isolate TN352 has a mutation at codon 268 in the cytochrome *bc*<sub>1</sub> gene**

The blood sample was obtained at the outpatient department of the Tropical Institute in Munich from a 28 year old male traveller returning from Mali to Germany. After developing signs of malaria and a positive result of the Malaquick<sup>®</sup> rapid diagnostic test he took a complete course of Malarone<sup>®</sup> complying with dose and concomitant food administration and recovered. Four weeks later he relapsed and presented with fever and 1.5% parasitaemia of *P. falciparum* asexual forms. The patient was successfully treated with Riamet<sup>®</sup> (lumefantrine and artemether).

The DNA was extracted and the parasite cytochrome *bc*<sub>1</sub> coding region was cloned and sequenced together with the DNA samples of the *in vitro* cultures. The sequence showed a unique single base change from TAT to TCT at the codon 268 resulting in an amino acid change from tyrosine to serine (Y268S).

### **Development of a PCR-RFLP method to detect mutations at codon 268 of the parasite cytochrome *bc*<sub>1</sub> coding region that are associated with resistance to atovaquone/proguanil *in vivo***

A nested PCR was designed with an outer product of 939 bp amplified with primer CYTb1 and CYTb2. In order to have restriction sites that are able to distinguish the wild type from the two reported mutations at codon 268, three new primers have been designed (see Table 1). The second amplification rounds used following pairs of primers: CYTb3/CYTb5, CYTb2/CYTb6 and CYTb2/CYTb6. The products was digested with NsiI, AlwNI and SspI, respectively (see Table 2 for details). Primer sequences are given in table 1 and fragment sizes after enzyme digest in table 2. NsiI cuts the wild type TAT, AlwNI cuts the mutation type TCT, and SspI cuts all except the mutation type AAT. The resulting pattern after restriction enzyme digest and gel electrophoresis is shown at Figure 1. Fragment sizes differ in 24 to 25 base pairs and require therefore a high resolution gel and optimised running conditions of the electrophoresis.

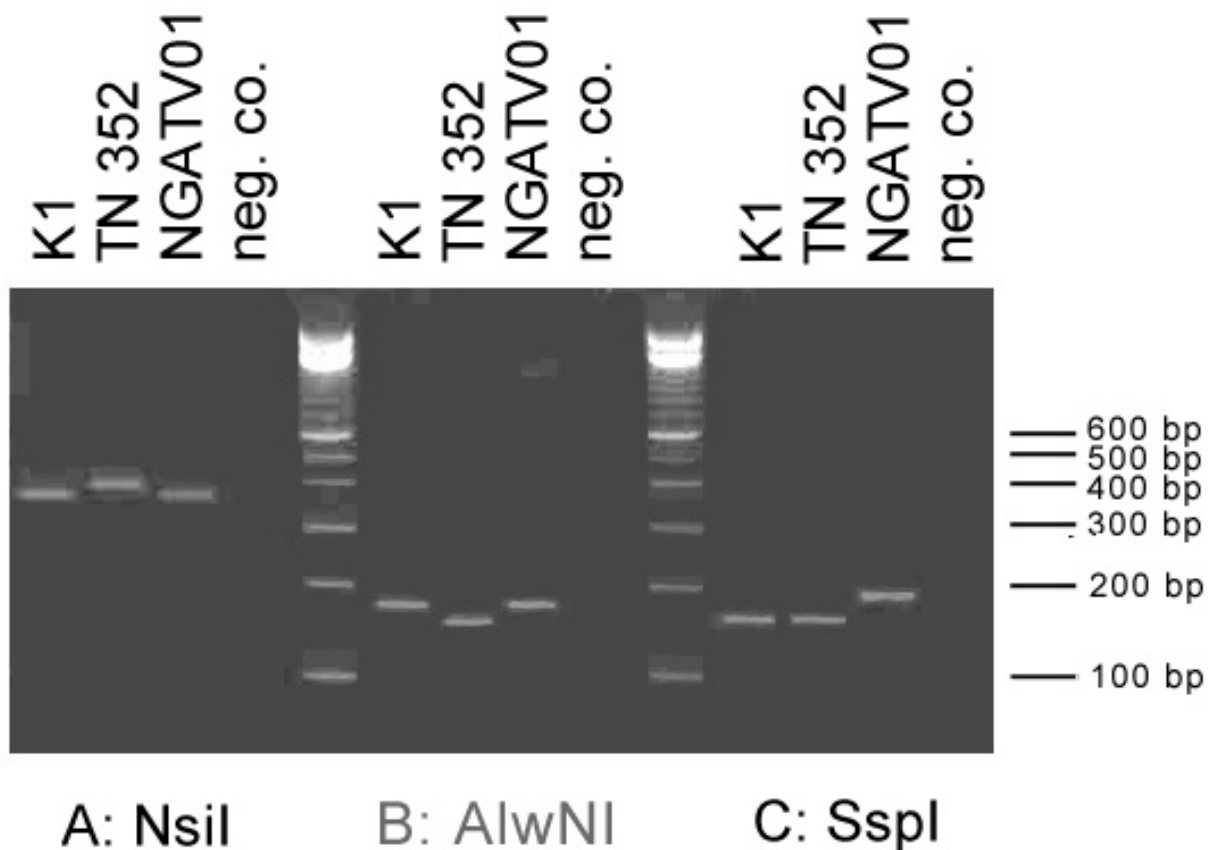
### **Discussion**

Atovaquone is an antimalarial substance that leads to an effective clearance of initial parasitaemia, but shows high recrudescence rates when used in monotherapy [1]. In order to improve the therapeutic response and slow the development of drug resistance atovaquone is used in a fixed combination with proguanil (Malarone<sup>®</sup>). Atovaquone acts by collapsing the mitochondrial membrane potential and therefore inhibiting parasite respiration [10]. Single point mutations at the cytochrome *bc*<sub>1</sub> gene of *P. falciparum* have been associated with atovaquone resistance [15]. We investigated sequence changes on the *P. falciparum*

cytochrome *bc*<sub>1</sub> gene after exposure to atovaquone alone and in combination with cycloguanil, the active metabolite of proguanil. Cultures that survived atovaquone concentrations from  $2 \times 10^{-8}$  up to  $10^{-7}$  M alone or in combination with cycloguanil showed a single point mutation at codon 133 (ATG to ATT) resulting in an amino acid change from methionine to isoleucine (M133I). When subjected to  $2 \times 10^{-7}$  M atovaquone in the culture medium, the parasites showed an additional mutation at codon 271 (TTA to TTC) resulting in an amino acid change from leucine to phenylalanine (L271F). No second mutation was found for parasites that survived  $2 \times 10^{-7}$  M atovaquone in combination with cycloguanil. The M133I mutation has also been reported from an atovaquone resistance line of *P. berghei* [14] but caused by a change from ATG to ATA. A mutation at codon 271 (TTA to GTA) has been described already for atovaquone resistant *P. yoelii* lines (L271V) in combination with the K272R mutation [13]. We found the mutation L271F in addition to the M133I change. Tandem mutations composed of M133I (ATG to ATA) with a second change in the region of codon 272 to 280 at the cytochrome b gene have been reported by Korsinczky et al. [15] after similar *in vitro* selection of mutations. Similar to our findings, he described an initial mutation at codon 133 resulting in a steric alteration at the putative atovaquone binding site. While the different tandem mutations do not affect predicted binding sites, they may enhance the alteration by the 133 substitution.

As we could demonstrate, the treatment of the K1 parasite culture with both drugs at sub-curative dosages did not prevent the development of the M133I mutation. Since the laboratory strain K1 is resistant to cycloguanil, a protective effect of the combination can be expected at higher doses of cycloguanil and has been shown for  $2 \times 10^{-7}$  M atovaquone in combination with cycloguanil. The influence of proguanil on the development of resistance to Malarone<sup>®</sup> *in vitro* has not been investigated. As *in vitro* study showed, acts proguanil compared to cycloguanil by distinct activities and has a synergistic effect in combination with atovaquone [18,19].

Sequencing of a *P. falciparum* clone derived from an *in vivo* treatment failure with Malarone<sup>®</sup> detected a single point mutation at codon 268 of the cytochrome b gene. The change of TAT to TCT resulted in an amino acid substitution from tyrosine to serine (Y268S). The same mutation has been described before in a Thai patient with recrudescence after atovaquone and pyrimethamine treatment and a 10,000 fold increase of IC<sub>50</sub> to atovaquone [1,15]. Fivelman et al. [8] showed a case of Malarone<sup>®</sup> resistance *in vivo* with 800 fold increase in IC<sub>50</sub> to atovaquone and a change to AAT at codon 268 resulting in a substitution to asparagine (Y268N). Furthermore the Y268S mutation has also



**Figure 1**

**Restriction digest method for detecting codon 268 mutations of cytochrome bc1** **A:** 384 bp amplification product with the primer pair CYTb3/CYTb5, digested by NsiI, K1 wild type (TAT) and AAT mutation (NGATV01) are cut (359 bp); **B:** 171 bp amplification product with the primer pair CYTb2/CYTb6, digested by AlwNI, only TN352 with the TCT mutation is cut to a 147 bp product; **C:** 174 bp amplification product with the primer pair CYTb2/CYTb7, digested by SspI, TAT wild type (K1) and TCT mutation (TN352) are digested (150 bp), while AAT mutation (NGATV01) remains uncut.

been identified in blood samples of a *P. falciparum* infected traveller returning from Cameroon to Denmark who failed Malarone<sup>®</sup> therapy (personal communication, Kim David). In all cases, the substitution of the bulky Tyr268 by less bulky amino acids in the region of the ubiquinol oxidation site might affect the fit and binding of the drug.

The mutation at codon 133, although observed *in vitro*, apparently does not influence development of *in vivo* resistance to atovaquone/proguanil. However, the three cases of confirmed atovaquone/proguanil resistance came from West Africa where Malarone<sup>®</sup> is not widely used yet. Increasing drug pressure with atovaquone/proguanil in

endemic areas may also select for the M133I mutation. Investigations on atovaquone resistant K1 clones *in vitro* found for the double amino acid mutation (M133I and G280D) 5 to 9% loss of fitness compared to the sensitive clone, but no detectable loss of fitness for the single mutation M133I [18].

For the development of a diagnosis test for resistance to atovaquone/proguanil we focused on the two mutations at codon 268 of the parasite cytochrome bc<sub>1</sub>. Out of four molecular analysed treatment failures to date with either atovaquone/proguanil or atovaquone/pyrimethamine, all four samples showed mutations at codon 268. Both ami-

no acid changes, Y268N and Y268S, resulted in extremely high increase of the IC<sub>50</sub>. Despite the low number of cases described to date indicate these results the relevance of codon 268 polymorphisms as potential resistance marker. To achieve a higher sensitivity, a nested PCR was designed with 3 different second rounds. Primers that anneal near the target mutations have been changed in one or two nucleotides in order to create restriction sites. The TAT wild type and the TCT mutation is demonstrated by the direct cut of the PCR product, while the AAT mutation is indicated when SspI does not cut. This might be a disadvantage of the method. However, with consequent use of positive and negative controls this is a precise method for the detection of codon 268 mutations.

### Conclusions

Mutations at codon 268 of the parasite cytochrome bc<sub>1</sub> gene are associated with atovaquone/proguanil treatment failure *in vivo* and can be used as possible resistance marker. Further work on more field isolates is required to prove the fundamental role of codon 268 mutations to govern drug resistance in the parasite. This new protocol permits the screening of patient samples from different malaria endemic regions and will be a useful tool in the context of TropNetEurop <http://www.tropnet.net>, a clinical network on imported infectious disease surveillance. Samples of returning travellers are collected within the network and examined continuously for molecular resistance markers [21]. Imported isolates will be analysed for codon 268 mutations to gain data on the prevalence of those mutations and their relevance for monitoring the further emergence of Malarone® resistance.

### Authors' contributions

BS carried out the *in vitro* cultivation of *P. falciparum* as well as the molecular genetic studies and drafted the protocol. MA participated in design and coordination of the molecular genetic studies. AS participated in cloning, sequencing and sequence alignment. TJ conceived of the study and participated in its coordination. All authors read and approved the final manuscript.

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