Amodiaquine resistance in *Plasmodium falciparum* malaria is associated with the *pfcrt* 72-76 SVMNT allele in Afghanistan

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Running title: AQ resistant malaria in Afghanistan

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Abstract

Mutations in *Plasmodium falciparum* genes *pfcrt* and *pfmdr1* are selected by amodiaquine treatment in Africa. To examine their importance in amodiaquine treated Asian parasites, we determined pre- and post-treatment genotypes in amodiaquine treatment failures from a clinical trial in Afghanistan. The *pfcrt* codon 72-76 haplotype SVMNT was present in all samples tested, before and after treatment. Amodiaquine did not clearly select for any *pfmdr1* genotype, but a novel mutation, *pfmdr1* N86F, was detected in four samples. We provide *in vivo* data to support the *in vitro* correlation between *pfcrt* SVMNT and increased resistance to the metabolite of amodiaquine.

Introduction

Amodiaquine (AQ), a 4-aminoquinoline related to chloroquine (CQ), has been used commonly as a monotherapy and now as a partner drug in artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *Plasmodium falciparum* malaria. In many countries, predominantly in Africa, the *in vivo* efficacy of AQ was found to be good even in the face of increasing CQ resistance (12). However, reports of AQ resistance have come from South America, Asia and East Africa (7, 8, 10).

Mutations in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) and multidrug resistance gene 1 (*pfmdr1*) have been associated with both CQ and AQ clinical resistance (13). The presence of the *pfcrt* 72-76 amino acid haplotype ‘SVMNT’ (Ser-Val-Met-Asn-Thr) correlates with high resistance to AQ metabolite desethylamodiaquine (DEAQ) in *in vitro* tests (14) and has been detected at a high
prevalence in parasite populations from Brazil, Papua New Guinea, Laos, Iran and India (9, 18). Clinical trials in East Africa have also demonstrated high in vivo resistance to AQ; here the parasites carried \textit{pfcrt} 72-76 haplotype CVIET, and \textit{pfmdr1} polymorphisms 86Y, 184Y and 1246Y were found to be selected after AQ treatment failure (5, 6, 11).

A clinical trial performed in Nangahar province, East Afghanistan, in 2002/03 to explore possible replacement treatments for CQ showed very poor efficacy of both CQ and AQ monotherapy (adequate clinical and parasitological response of 11% and 9% by day 42 respectively) (4). Our aim in this study was to evaluate pre and post-treatment samples from patients treated with AQ for \textit{pfcrt} and \textit{pfmdr1} mutations and to determine which, if any, polymorphisms are associated with AQ treatment failure in Afghanistan.

\textbf{Methods}

We analysed samples from AQ treated participants, collected during a clinical trial of AQ vs CQ vs sulphadoxine-pyrimethamine vs AQ+artesunate in East Afghanistan between October 2002 and January 2003. The clinical and parasitological results of drug efficacy have been reported elsewhere (4). \textit{Amodiaquine was supplied by Parke-Davis (Basoquin).} Ethical approval for the in vivo study and collection of samples for genotyping was given by the LSHTM Ethics Committee and locally by the Ministry of Public Health, Afghanistan. All enrolled participants gave informed consent.
83 trial participants received AQ monotherapy, 7 were excluded or lost to follow-up and 69 of the remaining 76 patients (91%) failed treatment, with parasites detected during the 42 day follow-up. We analysed available blood spots collected on filter paper pre-treatment (day 0, n=55) and on the 7th day or later on which parasites were detected by microscopy after treatment (day fail, n=42). Parasite DNA was extracted from blood spots as previously described (6).

Determination of polymorphism at \textit{pfcrt} codons 72-76 was performed using real-time PCR with double-labeled 26-mer oligonucleotide probes corresponding to the 3 most common \textit{pfcrt} 72-76 alleles (16). These are CVMNK (CQ sensitive), CVIET (CQ resistant) and SVMNT1 (CQ resistant, 7G8 type). The assay was run on a Corbett Rotorgene 3000 (Corbett Lifesciences, Qiagen, Germany).

Polymorphisms at codons 86, 184, 1034, 1042 and 1246 of the \textit{pfmdr1} gene were determined by PCR-sequence specific oligonucleotide probe assay (6) or by PCR and direct sequencing of amplicons using BigDye® Terminator v3.1 Cycle Sequencing Kits and analysis on an ABI 3730 sequencer (Applied Biosystems). The sequence chromatograms were analyzed using Chromas v2.75.

Changes in genotype prevalence between pre and post-treatment samples were assessed by two-tailed Fisher's exact test.

**Results**

The \textit{pfcrt} codon 72-76 haplotypes were successfully analyzed in 55 pre and 42 post treatment samples. All samples harboured the SVMNT allele. One day 0 sample
carried a mixed infection with SVMNT and the CQ and AQ sensitive CVMNK allele. CVMNK, however, was not detected after AQ treatment.

We investigated 5 polymorphic codons of *pfmdr1* in 83 samples (50 day 0 and 33 day fail). DNA was of insufficient quality to amplify and sequence all 5 sites from all available bloodspots. The prevalence of amino acids at codons 86, 184, 1034, 1042 and 1246 are shown in Table 1. Polymorphism was seen at codons 86 and 184 only; the sequence at codons 1034, 1042 and 1246 was wild type both before and after treatment with AQ.

Our sequence analysis revealed a novel *pfmdr1* mutation at codon 86. In comparison to 3D7, a change of 2 nucleotides has occurred at this codon from AAT, which codes for asparagine (N) to TTT which codes for phenylalanine (F). Therefore this polymorphism can be annotated N86F. It was observed in 1 day 0 and 3 day fail samples.

When *pfmdr1* codons were analyzed together 5 distinct haplotypes were found: NYSND, NFSND, YFSND, YYSND and FYSND. The prevalences of each in day 0 and day fail samples are shown in Figure 1.

The 3D7 laboratory isolate has *pfmdr1* haplotype NYSND and is defined as wild type. This haplotype was the most common among the pre-treatment and day of failure samples in Afghanistan, although the prevalence decreased from 58.0% (29/50) to 39.4% (13/33; P=0.12), respectively.
Discussion

We have found that a high prevalence of AQ malaria treatment failure in vivo is associated with *P. falciparum* parasites carrying the *pfcrt* 72-76 SVMNT allele in Jalalabad, Afghanistan. A modest increase in the prevalence of *pfmdr1* 86Y and of 184F was observed after AQ treatment failure, but these changes were not statistically significant. There is therefore no evidence of AQ selection on *pfmdr1* in this population of parasites, in which almost all AQ-treated individuals had recurrent infections within 42 days of follow-up. We note that the samples tested were collected more than 7 years ago and it is not known if *pfcrt* 72-76 SVMNT remains the predominant circulating allele in Afghanistan.

Genetic diversity in this parasite population is low and the variation within the polymorphic *msp2* gene sequence was insufficient to distinguish between recrudescent and new infections. In 101 randomly selected baseline (day 0) samples, 10 different alleles of *msp2* were detected, although 1 of these was found in almost half of all samples (data not shown). We assume that all recurring parasites were the result of recrudescent infections as the transmission intensity in the study area during the period of follow up was low, this being the start of winter season, and it was unlikely that trial participants would have received a second infectious bite within 42 days (N. Durrani, pers comm.). However, we recognize that it is a limitation of the study that we could not positively identify recrudescences from new infections using genotyping.
Our findings provide direct in vivo evidence that the pfcrt 72-76 allele SVMNT is sufficient to confer AQ resistance, independent of pfmdr1 genotype. In contrast, the CQ resistant CVIET allele is necessary, but insufficient to confer AQ resistance in vivo (5, 6, 11). In vitro studies of natural P. falciparum isolates, laboratory reference strains, progeny of genetic crosses and parasites transfected with allelic replacements support this conclusion (14, 15).

On the basis of transfections and in vitro drug tests described by Sidhu et al (15), Warhurst (17) observed that IC50 values for CQ in the presence of verapamil showed correlation with IC50 values for DEAQ. Since both CQ/verapamil and DEAQ effects were correlated with the hydrophobicity of pfcrt amino acids 72-76, he predicted that clinical failure of amodiaquine treatment would be associated with parasites carrying the relatively hydrophilic 72-76 SVMNT.

Recent molecular studies have found that pfcrt SVMNT haplotypes predominate in parts of the world historically reporting in vivo AQ resistance. Indeed many of these countries first introduced AQ as an antimalarial in the late 1940s, such as India, the Philippines and Brazil (14), raising the conjecture that AQ rather than CQ drug pressure selected for the SVMNT haplotype.

In contrast, in many areas where parasites carry the CQ-selected pfcrt CVIET haplotype, such as across sub-Saharan Africa, AQ remains a relatively effective drug and is now employed as the partner drug to artesunate as first line antimalarial regimen in 19 countries. To our knowledge, pfcrt SVMNT carrying malaria parasites have been reported only twice in Africa, in Ghana in 1996/7 (9) and in Tanzania in
It is clearly of some concern that such parasites would have a survival advantage in areas where AQ is in use.

In parts of east Africa, AQ seems to work less well than in west Africa; clinical trials in Kenya and Tanzania have shown a high proportion of *in vivo* AQ treatment failures (5, 10). In the Tanzanian trial the predominant *pfcrt* 72-76 haplotype was CVIET (6) and in both trials, significant selection of *pfmdr1* genes encoding 86Y, 184Y and 1246Y occurred after AQ monotherapy. We postulate that in the presence of high prevalences of *pfcrt* CVIET carrying parasites, AQ can still be effective and that *pfmdr1* mutations are required for the development of clinically significant AQ resistance. It is clear however that the correlation between *pfcrt* and *pfmdr1* loci and AQ efficacy is not clear-cut and varies across different geographical settings. Further beneficial modulation of the prevalence of resistant *pfmdr1* haplotypes may occur due to widespread use of artemether-lumefantrine in sub-Saharan Africa; this drug favours survival of the AQ-sensitive *pfmdr1* 86-184-1246 NFD haplotype (6).

The importance of the *pfmdr1* 86F allele with regard to AQ resistance, found in 4 samples, is not known. The same mutation was recently described in 2 isolates collected in Swaziland in 1999 (3). To our knowledge, this codon sequence has only been reported previously in laboratory isolates under *in vitro* mefloquine selection (2). Similar mutations may have been missed in studies where restriction fragment length polymorphism techniques were used to determine genotypes. The most parsimonious explanation of its appearance would be as a single nucleotide change from *pfmdr1* 86Y, rather than 2 nucleotide changes from the wild type *pfmdr1* 86N.
In this study the predominant \textit{pfmdr1} haplotype before and after AQ treatment failure was wild type NYSND, in contrast to our findings in Tanzania (6). Taken together, the results reported here, the \textit{in vitro} studies highlighted above and the predictions of Warhurst (17) strongly suggest that carriage of \textit{pfcrt} SVMNT alone is sufficient to predispose a parasite to be clinically highly AQ resistant and leads to the conclusion that ACTs with AQ as the partner drug should not be deployed in parts of the world where parasites with the SVMNT haplotype predominate.

**Acknowledgments**

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**Conflict of Interest**

The authors do not have a commercial or other association that might pose a conflict of interest.


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Table 1: Distribution of pfmdr1 polymorphisms in day 0 and day fail samples. Genotype changes occurred at codons 86 and 184 only. 86N (wildtype) was found at 82.0% in pre-treatment sample and 69.7% post-treatment (p=0.29). 184Y prevalence decreased from 62.0% pre-treatment to 54. post-treatment (p=0.65). None of the changes show statistically significant evidence of selection.
Figure 1: Prevalence of pfmdr1 haplotypes at amino acids 86, 184, 1034, 1042 and 1246 before and after treatment with AQ.