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Short Report: Polymorphisms in the *pfcrt* and *pfmdr1* Genes of *Plasmodium falciparum* and *in Vitro* Susceptibility to Amodiaquine and Desethylamodiaquine

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Abstract. The potential role of polymorphisms in the *pfcrt* and *pfmdr1* genes and *in vitro* susceptibility to amodiaquine and desethylamodiaquine were explored in 15 chloroquine-resistant Colombian *Plasmodium falciparum* isolates. Single nucleotide polymorphisms in the *pfcrt* gene, including a newly reported mutation (*S334N*), were seen in isolates with decreased susceptibility to amodiaquine and desethylamodiaquine. The lowest susceptibility found to amodiaquine was observed in an isolate carrying a *pfcrt* and *pfmdr1* Dd2-like haplotype, whereas a *pfcrt* haplotype related to the 7G8 Brazilian strain was found in a Colombian isolate with the lowest susceptibility to desethylamodiaquine. This exploratory study suggests that polymorphisms in the *pfcrt* and *pfmdr1* genes play a role in amodiaquine and desethylamodiaquine resistance and warrants further study.

The development and expansion of resistance to the mainstay antimalarials chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) are the major causes for the increased morbidity and mortality of *P. falciparum*. In response to the increasing resistance, combination therapy, and especially artemisinin-based combination therapy (ACT), is now increasingly advocated as first-line therapy.[1] The hypothesis is that a combination of drugs will achieve a more effective clinical and parasitologic cure, protect each other from the development of resistance, and reduce the overall rate of malaria. However, the choice of drugs is critical, especially bearing in mind the diversity and dynamics of *P. falciparum* and its possibility to develop different mechanisms of resistance.

The 4-aminoquinoline amodiaquine (AQ) is an antimalarial compound structurally and functionally related to CQ. It is more effective against CQ-resistant *P. falciparum* strains, and it is currently used in combination with artemisinin derivatives or SP for uncomplicated *P. falciparum* malaria, both in Africa[2,3] and South America (Colombia).[4] AQ is absorbed quickly in the gastrointestinal tract and metabolized in the liver by the cytochrome P450 isoform *CYP2C8*.[5] Its main metabolite is desethylamodiaquine (DEAQ), which seems to exert the main antimalarial effect because of its significantly longer elimination half-life.[6,7]

Several polymorphisms in two *P. falciparum* genes, *pfcrt* and *pfmdr1,* have been associated with resistance to 4-aminoquinoline antimalarial drugs.[8–10] Mainly *pfcrt* 76T, but also *pfmdr1* 86Y, has been involved in the development of CQ resistance and has also been associated with therapeutic failures after AQ treatment in Sudan, Kenya, and Nigeria.[11–13] In Colombia, where CQ-resistant *P. falciparum* is widespread but a more varied response to AQ has been found,[14,15] the *pfcrt* 76T allele seems to be presently fixed and ubiquitous, whereas the *pfmdr1* 86Y allele seems to be rare.[16,17]

In vitro studies have shown that probably through the *PFCRT* protein, the Ca^{2+}-transporter inhibitor drug verapamil (VP) may sensitize *P. falciparum* to CQ and to some extent to DEAQ but not to AQ.[18,19] These differences between CQ and AQ/DEAQ and between AQ and DEAQ raise the hypothesis that other polymorphisms, maybe in the *pfcrt* or *pfmdr1* genes, could mediate a decreased susceptibility to AQ and/or DEAQ.

To address this hypothesis and to further explore the resistance mechanisms against AQ/DEAQ, we analyzed the relationship of *pfcrt* and *pfmdr1* mutations in 15 Colombian *P. falciparum* isolates with their *in vitro* susceptibility to CQ, AQ, and DEAQ. Documented *in vitro* data from four *P. falciparum* reference strains (Dd2, HB3, 3D7, and 7G8) were also included in the analysis as comparators.[10,18,20]

The 15 *P. falciparum* culture adapted isolates, collected between 1999 and 2001 in three endemic areas along the Colombian Pacific Coast, were analyzed in a radioisotopic *in vitro* susceptibility assay for their response to CQ, AQ, and DEAQ.[21] The isolates were defrosted, adapted to continuous culture in supplemented RPMI-1640, and synchronized with sorbitol.[22–24] Ninety-six-well plates were coated with 50–3,200 nmol/L CQ and 5–320 nmol/L AQ and DEAQ after a 2-dilution factor (AQ and CQ were provided by the World Health Organization and DEAQ by the Walter Reed Army Institute). The final concentration of [^{3}H] hypoxanthine was 0.5 μCi/well and that of Albumax I was 0.5%.[21] The samples were incubated at 37°C for 48 hours, and each assay was done in duplicate. The counts per minute (cpm) were measured in a scintillation counter (LS7500; Beckman Instruments, Palo Alto, CA), and the IC_{50}s were calculated using the PROBIT program in SPSS 7.5 for Windows 98 (SPSS, Chicago, IL).

The capacity of VP (V4629-1G; Sigma, St. Louis, MO) to sensitize *P. falciparum* to CQ, AQ, and DEAQ was tested in the isolates TA7519, CA2855, and Dd2 strain, according to the recently developed ELISA-histidine rich protein II (HRP2)–based assay.[25] *In vitro* cultured parasites were diluted to an initial parasitemia of 0.05% and aliquoted into microculture 96-well plates pre-dosed with ascending concentrations of 16–2,020 nmol/L CQ, 2–270 nmol/L AQ, and 6–780 nmol/L DEAQ ± VP 0.8 μmol/L. After incubation at 37°C for 72 hours, the samples were freeze-thawed, transferred, and processed in pre-coated ELISA plates (Cellabs, New South Wales, Australia) for spectrophotometric analysis (Multiskan EX; Thermo Labsystems, Helsingfors, Finland) of parasite growth. The IC_{50} values were determined using HN-NonLin V1.05 Beta H. Noedl 2001 (http://malaria.farch.net). All samples were done in quadruple.

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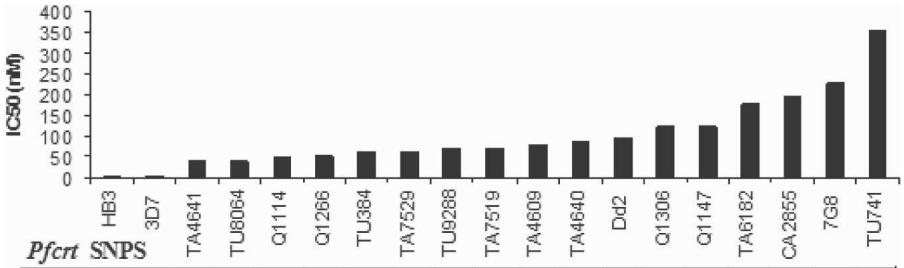
For the full sequencing of the *pfcrt* gene, total RNA and cDNA were obtained with Trizol LS (Invitrogen, Carlsbad, CA) and SUPERSCRIPT II RT (Invitrogen), respectively. Using the sequence of the HB3 strain (GenBank no. AF233068), primers were designed to amplify three overlapping fragments representing the complete *pfcrt* sequence (first block of 437 bp, second block of 409 bp, and third block of 593 bp). After a nested polymerase chain reaction (PCR) strategy, all blocks were sequenced (ABI 3700 Capillary DNA Sequencer; Cybergene AB, Huddinge, Sweden) and bioinformatically analyzed (Chromas V2.75, BLAST, Translate, LALING, and Clustal W). For the *pfmdr1* single nucleotide polymorphism (SNP) analysis, a PCR-restriction fragment length polimorphism (RFLP) strategy[26] was used to identify SNPs in positions N86Y, F184Y, S1034C, D1042N, and D1246Y (more details about these strategies can be requested from the authors). For the quantification of the *pfmdr1* gene copy number, a TaqMan real-time PCR-based protocol (Applied Biosystems, Fresno, CA) was used as published elsewhere.[27] Copy numbers were rounded to the nearest integer. This study was approved by the Ethical committees of CIDEIM and Universidad del Valle in Cali, Cali, Colombia.

In all 15 Colombian isolates, the IC_{50} values for CQ (114.58–415.53 nmol/L, data not shown) were above the WHO recommended cut-off for *in vitro* CQ resistance (IC_{50} > 100 nmol/L). More variations in IC_{50} values were observed for both DEAQ (38.31–355.8 nmol/L; Figure 1) and AQ (9.63–64.61 nmol/L; Figure 2).

VP was able to considerably decrease the IC_{50} values in Dd2, CA2855, and TU7519 for CQ from 340.1 to 129.7; 636.9 to 182.3; and 551.7 to 70.9 nmol/L for each sample, respectively. Similarly, VP reduced the IC_{50} values for DEAQ from 32 to 12.8; 119.7 to 26.6; and 62.9 to 12.2 nmol/L, respectively. In contrast, VP did not modify the *in vitro* response to AQ: 13.1 to 11.0; 26.5 to 28.5; and 22.5 to 22.2 nmol/L, respectively.

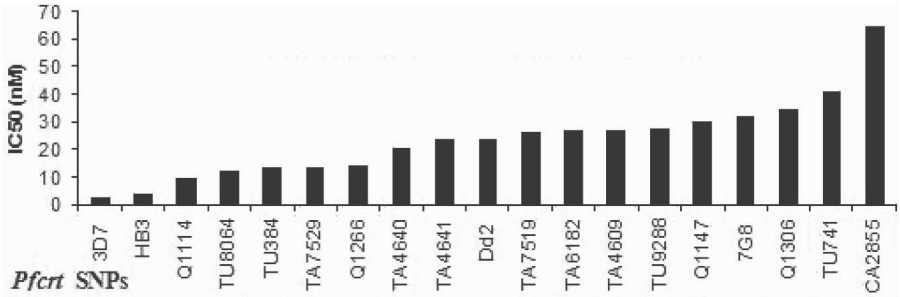
DNA sequencing of the *pfcrt* gene was completely successful in 12 of 15 samples. Three samples were uncertain in the 371 position. A total of 15 SNPs in 12 positions in the *pfcrt* gene were identified (Figures 1 and 2). All 15 Colombian isolates carried the 76T and 220S mutant haplotype. In codons 72–76 of the *pfcrt* gene, the CVMET haplotype was seen in 13 of 15 isolates. The isolate TU741 carried a CVMNT haplotype, which has also been found in Ecuador and Peru.[10,28] The isolate CA2855 showed a CVIET Dd2-like haplotype extending the reports of the presence of this Southeast Asian CQ-resistant haplotype in South America.[10,28] The SVMNT haplotype characteristic of the 7G8 CQ-resistant strain from Brazil and reported in *P. falciparum* populations from Papua New Guinea,[29] which has been proposed to be associated with DEAQ resistance,[30] was not detected in any Colombian sample.

The SNP 97Q that to this date has been only reported in Colombia was found in the majority (13/15) of the parasites.[10,17] In the TU741 isolate, a not previously described SNP, 334N, was identified. The SNP 333S was found in two isolates (TA4640 and TA6182); this SNP was previously found in Cambodia.[31] In summary, we identified four *pfcrt* haplotypes among the fully sequenced samples (amino acids 72, 74–76, 97, 220, 271, 326, 333, 334, 356, and 371; SNPs are underlined): CMETQSQNTSIT (8/12), CMETQSQNSSII (2/



Pfcrt SNPs	HB3	3D7	TA4641	TU8064	Q1114	Q1266	TU384	TA7529	TU9288	TA7519	TA4609	TA4640	Dd2	Q1306	Q1147	TA6182	CA2855	7G8	TU741
72	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S	C
74	M	M	M	M	M	M	M	M	M	M	M	M	I	M	M	M	I	M	M
75	N	N	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	N	N
76	K	K	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
97	H	H	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	H	H	H
220	A	A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
271	Q	Q	E	Q	Q	E	Q	Q	Q	Q	Q	Q	E	Q	Q	Q	E	Q	Q
326	N	N	N	N	N	N	N	N	N	N	N	N	S	N	N	N	S	D	D
333	T	T	T	T	T	T	T	T	T	T	T	S	T	T	T	S	T	T	T
334	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	N
356	I	I	I	I	I	I	I	I	I	I	I	I	T	I	I	I	T	L	L
371	R	R	--	T	--	--	T	T	T	T	T	I	I	T	T	I	I	R	R
Pfmdr1 SNPs AND COPY NUMBERS (CN)																			
86	N	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N
184	F	Y	F	F	F	F	F	F	F	F	F	F	Y	F	F	F	Y	F	F
1034	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	C	S
1042	D	N	D	D	D	D	D	D	D	D	D	D	N	D	D	D	N	D	D
1246	D	D	Y	D	D	Y	D	D	Y	D	D	Y	D	Y	Y	Y	D	Y	Y
CN	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1

FIGURE 1. *In vitro* susceptibility to desethylamodiaquine and genetic profile in the *pfcrt* and *pfmdr1* genes of *P. falciparum* reference strains and Colombian isolates. SNPs are shadowed. NS; no sequence data.



Pfcrt SNPs	3D7	HB3	Q1114	TU8064	TU384	TA7529	Q1266	TA4640	TA4641	Dd2	TA7519	TA6182	TA4609	TU9288	Q1147	7G8	Q1306	TU741	CA2855
72	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S	C	C	C
74	M	M	M	M	M	M	M	M	M	I	M	M	M	M	M	M	M	M	I
75	N	N	E	E	E	E	E	E	E	E	E	E	E	E	E	N	E	N	E
76	K	K	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
97	H	H	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	H	Q	H	H
220	A	A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
271	Q	Q	Q	Q	Q	Q	E	Q	E	E	Q	Q	Q	Q	Q	Q	Q	Q	E
326	N	N	N	N	N	N	N	N	N	S	N	N	N	N	N	D	N	D	S
333	T	T	T	T	T	T	T	S	T	T	T	S	T	T	T	T	T	T	T
334	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	N	S
356	I	I	I	I	I	I	I	I	I	T	I	I	I	I	I	L	I	L	T
371	R	R	--	T	T	T	--	I	--	I	T	I	T	T	T	R	T	R	I
***Pfmdr1* SNPs AND COPY NUMBERS (CN)**																			
86	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	Y
184	Y	F	F	F	F	F	F	F	F	Y	F	F	F	F	F	F	F	F	Y
1034	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	C	S	S	S
1042	N	D	D	D	D	D	D	D	D	N	D	D	D	D	D	D	D	D	N
1246	D	D	D	D	D	D	Y	Y	Y	D	D	Y	D	Y	Y	Y	Y	Y	D
CN	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1

FIGURE 2. *In vitro* susceptibility to amodiaquine and genetic profile in the *pfcrt* and *pfmdr1* genes of *P. falciparum* reference strains and Colombian isolates. SNP are shadowed. NS; no sequence data.

12; GenBank no. DQ156109), CMNTHSQDTNLR (1/12; GenBank no. DQ156108), and CIETHSESTSTI (1/12).

Pfmdr1 analyses were successful in all Colombian isolates. Three *pfmdr1* haplotypes (amino acids 86, 184, 1034, 1042, and 1246) were identified: NFSDD (6/15), NFSDY (8/15), and YYSND (1/15). All tested Colombian isolates had one *pfmdr1* gene copy (Figures 1 and 2).

The high IC_{50} values for CQ and the more varying IC_{50} values for AQ and DEAQ, as well as the presence of *pfcrt* 76T and 220S in all Colombian samples, are in line with the high level of CQ resistance and the more various therapeutic response to AQ in Colombia. The VP results with sensitization for CQ and DEAQ but not for AQ confirm previously published data[18,19] and suggest a mechanism of response to AQ less dependent on the *pfcrt* gene compared with DEAQ.

The TU741 isolate showed the highest IC_{50} value for DEAQ (355.8 nmol/L) and the second highest for AQ (41 nmol/L). TU741 was the only isolate with a *pfcrt* 72–76 CVMNT haplotype. TU741 also showed several genetic alterations not present in any of the other studied Colombian isolates, namely the *pfcrt* 326D and 356L SNPs also seen in the clone 7G8 (*pfcrt* 72-76 SVMNT), with the second highest IC_{50} value for DEAQ (229,3 nmol/L), as well as *pfcrt* 334N. This suggests that the haplotypes *pfcrt* 72–76 SVMNT/CVMNT, 326D, 334N, and 356L might be associated with DEAQ resistance. The CA2855 isolate showed the highest IC_{50} for AQ (64.6 nmol/L) and the third highest to DEAQ (198.1 nmol/L). It was the only isolate carrying a Dd2-like SNP haplotype in both *pfcrt* and *pfmdr1*. However, CA2855 did have one copy of *pfmdr1* compared with three in Dd2 (Figure 2). In addition, CA2855 was identified as FC27 and Dd2 as IC1 families in the *msp2* gene (data not shown).

The presence of *pfmdr1* 86Y, 184Y, and 1042N in this isolate is consistent with recent *in vivo* data from Kenya where *pfmdr1* 86Y was selected among recrudescent infections but not among new re-infections after AQ monotherapy,[12] suggesting that this haplotype is a potential factor for AQ resistance. However, Dd2 with the same haplotype had a lower IC_{50} value for AQ. Maybe this difference can be explained by its multicopied *pfmdr1* gene or other genes involved in AQ resistance.

A visual analysis of the sequence data along with the IC_{50} values of DEAQ (Figure 1) and AQ (Figure 2) showed an accumulation of SNPs in the 3′ region of the *pfcrt* open reading frame (ORF). There was also a tendency of the *pfmdr1* 1246Y SNP toward higher IC_{50} values for AQ. A decrease in the hydrophobicity, volume of lateral chains of the amino acids, and changes in the charges of the *PFCRT* protein have been predicted to explain the mechanism by which these SNPs could decrease the efficacy of AQ.[30]

This exploratory study supports the hypothesis that AQ and DEAQ resistance might be associated with different combinations of polymorphisms in the *pfcrt* and *pfmdr1* genes. The *pfmdr1* copy number seems not be involved in 4-aminoquinolines resistance, which should be explored further.

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