Am. J. Trop. Med. Hyg., 69(2), 2003, pp. 184–187

SHORT REPORT: ASSOCIATION BETWEEN CHLOROQUINE AND AMODIAQUINE RESISTANCE AND ALLELIC VARIATION IN THE *PLASMODIUM FALCIPARUM* MULTIPLE DRUG RESISTANCE 1 GENE AND THE CHLOROQUINE RESISTANCE TRANSPORTER GENE IN ISOLATES FROM THE UPPER NILE IN SOUTHERN SUDAN

EDWIN O. OCHONG', INGRID V. F. VAN DEN BROEK, KEES KEUS, AND ALEXIS NZILA

Kenya Medical Research Institute, Wellcome Trust Collaborative Program, Médecins sans Frontières-Holland, South Sudan Section, Nairobi, Kenya; Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, United Kingdom

Abstract. Amodiaquine, a 4-aminoquinoline compound, is being considered as an alternative to chloroquine and pyrimethamine/sulfadoxine where resistance in *Plasmodium falciparum* to both drugs has been selected. Although amodiaquine is more potent than chloroquine, its effectiveness is reduced in areas where chloroquine resistance is high. We report an association of the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene, two chloroquine resistance markers, with chloroquine and amodiaquine efficacy *in vivo* in southern Sudan. The data show that the allele of the *pfcrt* gene with a lysine to threonine change at codon 76 is strongly associated with both chloroquine and amodiaquine resistance. No such association was observed with the *pfmdr1* gene.

In response to the spread of resistance to chloroquine (CQ) and pyrimethamine/sulfadoxine (Fansidar®; F. Hoffmann-La Roche, Basel, Switzerland), amodiaquine (AQ) is now being considered as an alternative option for the management of uncomplicated *Plasmodium falciparum* malaria in Africa.[1,2] Although this drug remains effective in areas of substantial CQ resistance,[3–6] the two drugs are chemically related and several clinical[1,2,7] and *in vitro*[8,9] reports have shown cross-resistance between CQ and AQ or the active metabolite of AQ.

Many studies have been devoted to understanding the mechanism of CQ resistance.[10.] Point mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene and, to a lesser extent, in the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene are associated with CQ resistance. Polymorphism in the *pfcrt* gene has been reported to correlate with CQ resistance.[11,12] Among the amino acid changes in this protein, the lysine to threonine change at position 76 (pfcrt 76T) is the most strongly associated with CQ resistance both *in vivo* and *in vitro*.[11,12] Recently, transfection of the *pfcrt* gene has clearly demonstrated the role of this mutant allele in CQ resistance *in vitro.*[13,14] However, in semi-immune populations, the value of this mutation for predicting clinical outcomes after CQ treatment has not been consistent.[15,16] The point mutation of asparagine to tyrosine at codon 86 in the *pfmdr1* gene (pfmdr1 86Y) has been associated with CQ resistance in some studies,[17,18] but not in others.[19,20]

The molecular mechanisms of CQ and AQ cross-resistance have not yet been addressed, but the similarity of their chemical structures, their likely common mode of action,[21,22] and some apparent cross-resistance suggest that molecular markers selected as a function of CQ use might also compromise effectiveness of AQ. We report the impact of mutant alleles pfcrt76T and pfmdr1 86Y on the clinical efficacy of AQ and CQ in southern Sudan, an area where CQ efficacy is still at high levels.

We analyzed samples collected during a clinical trial of efficacy of antimalarial agents in southern Sudan between June and December 2001.The study was reviewed and approved by the Ethical Committee of Médecins sans Frontières-Holland (Amsterdam, The Netherlands). Local authorities and the Sudanese People's Democratic Front/counterpart agreed with the study and helped to notify the population. Blood collected by finger prick (50 μL) was spotted onto filter paper, air-dried, and stored in plastic bags with silica gel at ambient temperature**.** Parasite genomic material was prepared using the methanol procedure described elsewhere.[23] To detect a single base change at codon 76 of pfcrt and codon 86 of pfmdr1, we used the polymerase chain reaction (PCR)–restriction enzyme protocol described in detail by Professor Christopher Plowe (University of Maryland, Baltimore, MD; Web site: http://medschool.umaryland.edu/cvd/2002_pcr_asra.htm).

The detailed clinical results of CQ and AQ efficacy in Sudan have been presented elsewhere.[24] Briefly, 104 and 101 patients were treated with CQ (10 mg/kg on day 0, 10 mg/kg on day 1, and 5 mg/kg on day 2) and AQ (10 mg/kg on day 0, 10 mg/kg on day 1, and 5 mg/kg on day 2), respectively. Of these, 14 (13.5%) of 104 and 7 (6.9%) of 101 had positive parasitemias within 14 days after treatment and were scored as CQ resistant and AQ resistant, respectively: these are parasitologic failures. Those whose blood samples were negative 14 days after treatment were scored as an adequate parasitologic response or harboring sensitive isolates. To test whether polymorphisms in pfcrt and pfmdr1 are associated with the CQ and AQ resistance response, we genotyped pfcrt at codon 76 and pfmdr1 at codon 86 in isolates from four groups of patients: those showing an adequate parasitologic response to CQ (n = 28) and AQ (n = 39), and those who had *P. falciparum* parasites in their blood within 14 days after treatment with CQ (n = 13) and AQ (n = 6). All of these isolates were collected on the day of admission into the study before treatment was given.

The *pfcrt* and *pfmdr1* genes were successfully amplified in all isolates except for one from the AQ-resistant group (for pfcrt) and two from the CQ-sensitive group (for pfmdr1). The analysis of pfcrt showed that 93% (26 of 28) of the isolates from patients treated with CQ with an adequate parasitologic response were wild type (pfcrt 76K) and all 13 CQ-resistant isolates carried the mutant allele (pfcrt 76T). The same trend was observed in patients treated with AQ: 85% (33 of 39)

TABLE 1

Allelic polymorphisms of the pfcrt gene at codon 76 in samples collected before chloroquine and amodiaquine treatments*

	Chloroquine (CQ) treatment		Amodiaquine (AQ) treatment	
	CQ sensitive (n = 28)	CQ resistant (n = 13)	AQ sensitive (n = 39)	AQ resistant (n = 5)
pfcrt 76K (wild type)	26/28 (93%)	0	33/39 (85%)	0
pfcrt 76T (mutant)	2/28 (7%)	13/13 (100%)	6/39 (15%)	5/5 (100%)

* pfcrt = *Plasmodium falciparum* chloroquine resistance transporter (gene).

were wild type and 100% (5 of 5) carried the mutant allele (pfcrt 76T) in the sensitive and resistant groups, respectively (Table 1). The analysis of pfmdr1 showed that 92% (24 of 26) of the CQ-sensitive isolates, as well as 92% (36/39) of the AQ-sensitive isolates, were wild type (pfmdr1 86N). In contrast, the pfmdr1 86Y mutant allele was only found in 62% (8 of 13) and 50% (3 of 6) of the isolates that failed to respond to CQ and AQ, respectively (Table 2). When pfcrt and pfmdr1 were analyzed together, more than 80% of the CQ- and AQ-sensitive isolates carried the wild type pfcrt 76K-pfmdr1 86N genotype (Figure 1). However, the combination of mutant genotypes pfcrt 76T-pfmdr1 86Y was observed only in approximately 60% of both AQ- and CQ-resistant isolates.

This is the first report of the assessment of the impact of pfcrt markers on *in vivo* efficacy of AQ. Our data clearly demonstrate that the pfcrt allele at codon 76, the most common marker for CQ resistance,[11–14] is also associated with AQ resistance *in vivo*. Both CQ and AQ are 4-aminoquinoline agents, and several reports[21,22] have shown that these two drugs act in a similar manner against *P. falciparum* by inhibiting the polymerization of heme, the toxic byproduct of hemoglobin degradation. It has been suggested that AQ is more potent than CQ because of a higher accumulation of AQ in the digestive vacuole of the parasite.[21,22,25] Therefore, since their chemical structures and their mode of action are similar, one would expect that the selection of CQ resistance would impact on the efficacy of AQ. As a consequence, selection of markers for CQ resistance would have a bearing on AQ resistance; this is what our data clearly show. Overall, CQ and AQ both retain excellent efficacy in our study site, and under these circumstances, the pfcrt mutant could be used as a predictor of both CQ and AQ resistance.

Recently, transfection studies of pfcrt have shown that isolates expressing the mutant pfcrt 76T allele retain sensitivity to AQ while showing a reduced susceptibility to monodesethylamodiaquine (MDAQ), the active metabolite of AQ.[13] Therefore, the association between the mutant allele pfcrt 76T and AQ resistance we have found in *vivo* may reflect an association of this allele with the active metabolite MDAQ.

The pfmdr1 86Y allele is not as strongly associated with resistance as pfcrt76T. Indeed, we have found that only 62% of the CQ-resistant isolates and 50% of the AQ-resistant isolates harbor the mutant pfmdr1 86Y allele. The lack of association between this allele and CQ resistance has been reported in different malaria-endemic areas.[15,19,20] We have confirmed these observations in southern Sudan with CQ and also report the lack of association of this marker with AQ resistance.

In conclusion, our study shows that the mutant pfcrt 76T allele is correlated with CQ resistance as previously reported.[11,12] We also provide evidence that the selection of this allele could explain, at least partly, the cross-resistance observed between CQ and AQ *in vivo*. However, this study was carried out in an area where CQ is still very effective. Therefore, it remains to be seen if this pattern will also be observed in the many areas of Africa where CQ resistance is already at high levels.

Received February 10, 2003. Accepted for publication May 12, 2003.

Acknowledgments: We thank the malaria laboratory staff and clinic workers in Lankien, Sudan for their unremitting assistance in the project, as well as the local authorities for their cooperation, and the Nuer community in southern Sudan for participation in the study. We also thank the staff of the Médecins sans Frontières-Holland South Sudan Section and the Médecins sans Frontières-Holland headquarters in Amsterdam for advice, and Professor C. Sibley for advice during the PCR/genotyping studies.

Financial support: This work was supported by the National Institutes of Health (Fogarty International grant TW 01186) and the Wellcome Trust of Great Britain (grant no. 056769). Alexis Nzila was supported by the Wellcome Trust.

Authors' addresses: Edwin O. Ochong', Kenya Medical Research Institute, Wellcome Trust Collaborative Program, PO Box 43640, 00100 GPO, Nairobi, Kenya. Ingrid. V.F. van den Broek and Kees Keus, Médecins sans Frontières-Holland, South Sudan Section, PO Box 4064, Nairobi, Kenya. Alexis Nzila, Kenya Medical Research Institute, Wellcome Trust Collaborative Program, PO Box 43640, 00100 GPO, Nairobi, Kenya and Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, United Kingdom, Telephone: 254-2-271-0672, Fax: 254-2-271-1673, E-mail: anzila@wtnairobi.mimcom.net.

TABLE 2

Allelic polymorphisms of the $pfmdr_1$ gene at codon 86 in samples collected before chloroquine and amodiaquine treatments*

	Chloroquine (CQ) treatment		Amodiaquine (AQ) treatment	
	CQ sensitive (n = 26)	CQ resistant (n = 13)	AQ sensitive (n = 39)	AQ resistant (n = 6)
pfmdr1 N86 (wild type)	24/26 (92%)	5/13 (38%)	36/39 (92%)	3/6 (50.00%)
pfmdr1 Y86 (mutant)	2/26 (8%)	8/13 (62%)	3/39 (8%)	3/6 (50.00%)

* pfmdr = *Plasmodium falciparum* multiple drug-resistance (gene).

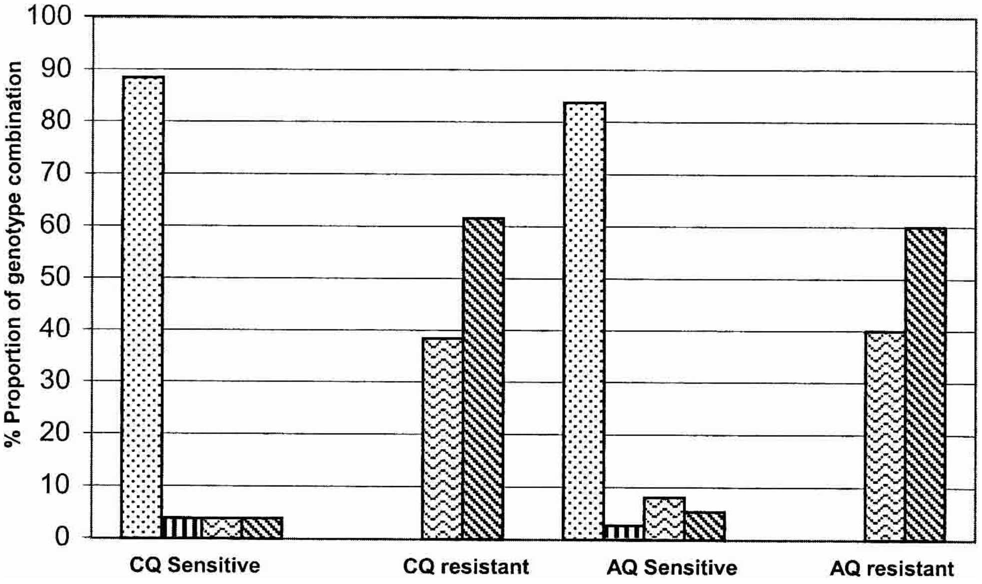

FIGURE 1. Allelic frequency of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene at codon 76 and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene at codon 86 in isolates collected before treatment with chloroquine (CQ) and amodiquine (AQ) in southern Sudan. The allelic combinations analyzed were wild type pfcrt 76K and wild type pfmdr1 86N; (**dotted bars**); wild type pfcrt 76K and mutant pfmdr1 86Y; (**vertically striped bars**); mutant pfcrt 76T and wild type pfmdr1 86N (**wavy-patterned bars**); mutant pfcrt 76 and mutant pfmdr1 86Y (**diagonally striped bars**).

REFERENCES

1. Schellenberg D, Kahigwa E, Drakeley C, Malende A, Wigayi J, Msokame C, Aponte JJ, Tanner M, Mshinda H, Menendez C, Alonso PL, 2002. The safety and efficacy of sulfadoxine-pyrimethamine, amodiaquine, and their combination in the treatment of uncomplicated *Plasmodium falciparum* malaria. *Am J Trop Med Hyg 67:* 17–23.
2. Bloland PB, Ruebush TK, 1996. Amodiaquine. *Lancet 348:* 1659–1660.
3. Staedke SG, Kamya MR, Dorsey G, Gasasira A, Ndeezi G, Charlebois ED, Rosenthal PJ, 2001. Amodiaquine, sulfadoxine/ pyrimethamine, and combination therapy for treatment of uncomplicated falciparum malaria in Kampala, Uganda: a randomised trial. *Lancet 358:* 368–374.
4. Van Dillen J, Custers M, Wensink A, Wouters B, van Voorthuizen T, Voorn W, Khan B, Muller L, Nevill C, 1999. A comparison of amodiaquine and sulfadoxine-pyrimethamine as first-line treatment of falciparum malaria in Kenya. *Trans R Soc Trop Med Hyg 93:* 185–188.
5. Brasseur P, Guiguemde R, Diallo S, Guiyedi V, Kombila M, Ringwald P, Olliaro P, 1999. Amodiaquine remains effective for treating uncomplicated malaria in west and central Africa. *Trans R Soc Trop Med Hyg 93:* 645–650.
6. Gorissen E, Ashruf G, Lamboo M, Bennebroek J, Gikunda S, Mbaruku G, Kager PA, 2000. *In vivo* efficacy study of amodiaquine and sulfadoxine/ pyrimethamine in Kibwezi, Kenya and Kigoma, Tanzania. *Trop Med Int Health 5:* 459–463.
7. White NJ, 1996. Can amodiaquine be resurrected? *Lancet 348:* 1184–1185.
8. Basco LK, Le Bras J, 1993. *In vitro* activity of monodesethylamodiaquine and amopyroquine against African isolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg 48:* 120–125.
9. Childs GE, Boudreau EF, Milhous WK, Wimonwattratee T, Pooyindee N, Pang L, Davidson DE Jr, 1989. A comparison of the *in vitro* activities of amodiaquine and desethylamodiaquine against isolates of *Plasmodium falciparum*. *Am J Trop Med Hyg 40:* 7–11.
10. Wellems TE, Plowe CV, 2001. Chloroquine-resistant malaria. *J Infect Dis 184:* 770–776.
11. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Wellems TE, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell 6:* 861–871.
12. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med 344:* 257–263.
13. Sidhu ABR, Pinard DV, Fidock DA, 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science 298:* 210–213.
14. Zhang H, Howard EM, Roepe PD, 2002. Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast. *J Biol Chem 277:* 49767–49775.
15. Thomas SM, Ndir O, Dieng T, Mboup S, Wypij D, Maguire JH, Wirth DF, 2002. *In vitro* chloroquine susceptibility and PCR analysis of pfcrt and pfmdr1 polymorphisms in *Plasmodium falciparum* isolates from Senegal. *Am J Trop Med Hyg 66:* 474–480.
16. Mayor AG, Gomez-Olive X, Aponte JJ, Casimiro S, Mabunda S, Dgedge M, Barreto A, Alonso PL, 2001. Prevalence of the K76T mutation in the putative *Plasmodium falciparum* chloroquine resistance transporter (pfcrt) gene and its relation to chloroquine resistance in Mozambique. *J Infect Dis 183:* 1413–1416.
17. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF, 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature 345:* 255–258.
18. Basco LK, Le Bras J, Rhoades Z, Wilson CM, 1995. Analysis of pfmdr1 and drug susceptibility in fresh isolates of *Plasmodium falciparum* from subsaharan Africa. *Mol Biochem Parasitol 74:* 157–166.
19. Haruki K, Bray PG, Ward SA, Hommel M, Ritchie GY, 1994. Chloroquine resistance of *Plasmodium falciparum*: further evidence for a lack of association with mutations of the pfmdr1 gene. *Trans R Soc Trop Med Hyg 88:* 694.
20. Pillai DR, Labbe AC, Vanisaveth V, Hongvangthong B, Pomphida S, Inkathone S, Zhong K, Kain KC, 2001. *Plasmo-*

dium falciparum malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. *J Infect Dis 183:* 789–795.

21. Bray PG, Mungthin M, Ridley RG, Ward SA, 1998. Access to hematin: the basis of chloroquine resistance. *Mol Pharmacol 54:* 170–179.
22. Ginsburg H, Famin O, Zhang J, Krugliak M, 1998. Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem Pharmacol 56:* 1305–1313.
23. Nzila A, Mberu E, Sulo J, Dayo H, Winstanley P, Sibley C, Watkins WM, 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *P. falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Antimicrob Agents Chemother 44:* 991–996.
24. Van den Broek IVF, Gatkoi T, Lowoko B, Nzila A, Ochong' E, Keus K, 2003. Comparison of chloroquine, sulfadoxine-pyrimethamine and amodiaquine efficacy to treat uncomplicated falciparum malaria in Upper Nile, South Sudan. *Trans R Soc Trop Med Hyg 97:* (in press).
25. Famin O, Ginsburg H, 2002. Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. *Biochem Pharmacol 63:* 393–398.