POLYMORPHISMS IN PF f CRT, PF MD R1, DH FR GENES AND IN VITRO RESPONSES TO ANTIMALARIALS IN PLASMODIUM FALCIPARUM ISOLATES FROM BANGUI, CENTRAL AFRICAN REPUBLIC

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Abstract. Drug resistance is probably the greatest challenge to most malaria-control programs. Given the limited resources for other malarial-control measures, rational drug used is crucial. Molecular markers for parasite resistance such as pf f CRT, pf md r1, and dh fr have the potential to be used in an integrated fashion to provide timely information that is useful to policy makers. Therefore, we evaluated polymorphisms in these genes from Plasmodium falciparum and their association with in vitro antimalarial drug resistance to 135 parasites samples collected in Bangui in 2004. For the dh fr gene, we found a strong association between the dh fr genotype and chemosensitivity to pyrimethamine. For the pf f CRT gene, we found that haplotypes with mutant-type alleles led to significant changes in the IC50 values for chloroquine, monodesethylamodiaquine, and quinine. We found no correlations for the pf md r1 gene. These findings suggest that a regular monitoring and screening for resistance markers for antifolates and for chloroquine could act as an adjunct to in vivo trials.

INTRODUCTION

The resistance to antimalarial drugs, especially chloroquine (CQ), of Plasmodium falciparum is one of the principal factors contributing to the worldwide increase in morbidity and mortality due to malaria.1,2 Different approaches have been developed to monitor the extent of antimalarial drug resistance and to determine the biologic mechanisms by which the parasite has evaded the action of the drug.

The susceptibility of P. falciparum to antimalarial drugs is usually assessed by therapeutic responses (in vivo test). This method has allowed the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies to be determined.3 However, the risk of loss of patients during long follow-up periods and the logistical demands of clinical response studies in endemic areas have led to the development of laboratory strategies for identifying anti-malaria drug resistance. Laboratory strategies include in vitro drug sensitivity tests and evaluation of molecular markers associated with drug resistance.4,5 Antimalarial drug sensitivity tested in vitro provides information on the frequency of the resistant phenotype among the populations of parasites being transmitted and the possible cross-resistance patterns of anti-malaria drugs.6 In vitro testing is also useful for evaluating the efficacy of new drugs and for investigating the biologic mechanisms of drug action and resistance.7,8 Although this method is useful, its application is limited because samples must be immediately cultured or cryopreserved for transport, parasites must be adapted to culture (which is laborious), and the tests use expensive and radioactive materials.9 Although more rigorous in vitro tests of culture-adapted isolates are more reproducible, the processes of freezing, thawing, and adaptation to culture also introduce the possibility of selecting subpopulations of parasites, so that the parasites ultimately assayed may be genetically and phenotypically unrepresentative of the original parasite population.

These limitations of in vivo and in vitro methods have led to the search for genetic markers of resistance. Potential molecular markers for parasite resistance to quinoline antimalarials are being identified. The association of CQ resistance and single nucleotide polymorphisms (SNPs) in the pf f CRT (CQR transporter) and pf md r1 (multidrug resistance 1) genes are presently well established.10–13 For example, the K76T mutation in pf f CRT gene was perfectly associated with in vitro resistance in all progeny of a genetic cross between chloroquine-sensitive and chloroquine-resistant parental clones and among a set of geographically diverse parasite isolates.14 Polymorphisms in pf md r1, which encodes the P. falciparum P glycoprotein homologue 1, modulate chloroquine resistance in mutant pf f CRT-harboring parasites in vitro,15 although their role in vivo has yet to be substantiated.10 The mutations most often cited as potential contributors to chloroquine resistance are pf md r1 N86Y and D1246Y. In the same way, several point mutations in P. falciparum dh fr and dh ps genes have been associated with sulfadoxine-pyrimethamine (SP) treatment failure in Africa.16–22 Univariate analysis have shown that the dh fr triple mutant S108N/N51I/C59R or the dh fr and dh ps double mutant A437G/K540E or the dh fr and dh ps quintuple mutant carrying all these 5 mutations are all associated with SP treatment failure in patients.19,21,22

In the Central African Republic (CAR), Plasmodium fal- ciparum resistance to CQ has been documented since 1983 and to SP since 1987.24 Since 2003, when the latest in vivo study was carried out,25 amodiaquine + SP combination has been used as interim first-line treatment, until the best alternative treatments, such as Artemisinin-based Combination Therapies (ACT), become available at low prices in the CAR. We have previously determined, in samples collected in 2004, the in vitro susceptibility of P. falciparum strains to antimalarials currently being used in the CAR (chloroquine, amodiaquine, quinine, and pyrimethamine), to antimalarials that will be available for future use in this region (artemisinin, halofantrine), and to prophylactic antimalarials (mefloquine, atovaquone, doxycycline).26 We have also previously determined the baseline frequency distribution of the mutant alleles of genes associated with antimalarial drug resistance (pf f CRT, pf md r1, dh fr, and dh ps).27

Here, we aim to complete these studies by determining the utility of the molecular markers such as pf f CRT, pf md r1, and
 dhfr in monitoring antimalarial drug resistance. We have estimated the association of these molecular makers with in vitro responses to antimalarial drugs (chloroquine, monodesethylamodiaquine, quinine, dihydroartemisinin, mefloquine, halofantrine, and pyrimethamine) in Plasmodium falciparum isolates from Bangui, Central African Republic.

METHODS

P. falciparum isolates. This study was conducted in Bangui, the capital of the CAR, between March and July 2004. Bangui is located beside the Oubangui river in the middle of central Africa, north of the Democratic Republic of the Congo (Geographic coordinates: 7 00 N, 21 00 E). The climate is tropical and rainfall is at its highest from April to November. The average temperature varies from 19–32°C. Malaria transmission occurs throughout the year, with peak transmission at the beginning and the end of the rainy season. Malaria is hyperendemic in this region and Plasmodium falciparum is the principal parasite. The prevalence of this parasite in children less than 5 years of age is 31.8%.28

We obtained 381 P. falciparum clinical isolates from symptomatic patients from the CAR attending several health centers in Bangui before the patients were treated. Venous blood samples from patients giving informed consent were collected in a tube coated with EDTA (Vacutainer® tubes, Becton Dickinson, Rutherford, NJ, USA). Giemsa-stained thin and thick blood smears were examined to check for mono-infection with P. falciparum and to determine parasite density. Blood samples with a parasite density > 0.1% were assayed in vitro within 8 hours of blood extraction. The patients were treated with amodiaquine-sulfadoxine-pyrimethamine combination or quinine, as recommended by the National Malaria Control Program in the CAR.

Ethical approval. As there is no National Ethics Committee in the CAR, study protocols were approved by the expert committee for antimalarial drug policy and the CAR Ministry of Health. Blood samples were obtained from patients after obtaining informed consent during routine malaria diagnosis.

In vitro drug sensitivity assay. Stock solutions of chloroquine, monodesethylamodiaquine, dihydroartemisinin, quinine, mefloquine, and halofantrine were prepared in methanol. The stock solution of pyrimethamine was prepared in ethanol. The final concentration in methanol and ethanol did not exceed 0.05%. Twofold (4-fold for pyrimethamine) serial dilutions of the stock solutions were prepared in distilled water. The solutions tested had concentrations ranging from 12.5–3200 nM for chloroquine, 25–3200 nM for quinine, 7.5–1920 nM for monodesethylamodiaquine, 0.25–64 nM for dihydroartemisinin, 0.25–32 nM for halofantrine, 1.5–400 nM for mefloquine, and 50–40,000 nM for pyrimethamine. Aliquots (20 µL) of each solution (at all concentrations tested) were transferred to 96-well tissue culture plates in triplicate.

The venous blood samples were washed 3 times in RPMI 1640 medium. The white blood cell interface was removed after each wash. The erythrocytes were then resuspended in a volume of complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% human serum (Serum AbCys®, France, lot no S0290954190), 25 mM HEPES buffer, and 25 mM sodium bicarbonate) giving a hematocrit of 1.5% and an initial parasitemia of 0.1–0.5%. Paraaminobenzoic acid-free and folic acid-free RPMI 1640 medium was used to assess the in vitro sensitivity to pyrimethamine. If the blood sample had a parasitemia greater than 0.5%, fresh uninfected erythrocytes were added to adjust the parasitemia to 0.3%. The technical procedure for the in vitro isospec microtest and calculation of 50% inhibitory concentrations (IC50) has been previously described.26

Extraction of DNA. The DNA template for PCR and the detection of mutant alleles was prepared from the whole blood sample. The blood was centrifuged and the erythrocytes were frozen at −20°C until extraction. Parasite DNA was extracted from 100 µL of thawed red blood cell pellets by treatment with 0.1M NaOH for 3 minutes at 100°C. The supernatant was collected and treated with 250 µL of lysing solution (0.1 M Tris HCl, Triton 100X, 1 M NaCl, SDS 10X, 0.5 M EDTA) and 20 mg/ml proteinase K for 1 hour at 37°C, and then extracted twice with phenol/chloroform (1:1). The DNA was then precipitated with ethanol, resuspended in 100 µL of distilled water, and stored at −20°C.

Amplification by polymerase chain reaction and detection of mutant alleles. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis were carried out for 3 genes (dihydrofolate reductase, dhfr gene at codons 16, 50, 51, 59, 108, 164; chloroquine resistance transporter, pfcr gene at codons 72, 74, 75, 76 and in multidrug resistance 1, pfmdr1 gene at codons 86 and 1246) to identify the presence of mutant alleles.

A detailed description of these methods is available on the Web at http://medschool.umd.edu/CVD/plowe.html.

All PCR amplifications were performed with Gene Amp PCR system 9700® thermocycler (Applied Biosystems®, Courtaboeuf, France) and Taq DNA Polymerase Promega® (Promega France, Charbonnieres, France). Five microliters of PCR products were incubated with restriction enzymes (New England Biolabs®, Beverly, MA, USA) according to manufacturer’s protocols in a 15-µL final reaction. Laboratory strains of P. falciparum served as controls (positive and negative) and were included in all PCR and enzyme digests procedures (purified genomic DNA from W2, HB3, and 3D7 reference strains from the Malaria Research Reference Reagent Resource Center, MR4/ATCC, Manassas, Virginia). A subset of 45 samples also was sequenced in Genopole at Institut Pasteur (Paris, France). Briefly, 50 µL of PCR products were purified using polyacrylamide P-100 Gel (Bio-Gel P-100, BioRad®, Marnes-la-Coquette, France) on 96-well plate filtration (Millipore®, St. Quentin en Yvelines, France). Evaluation of template quantity and quality was performed using agarose 1.2% gel and buffer TBE 0.5% (Invitrogen®, Cergy Pontoise, France) on Ektrofast micro gel system 96 wells (Fischer Scientific Labosis®, Ottawa, Canada) with DNA quantification kit (Molecular weight, Abgene®, Courtaboeuf, France). Sequencing reactions were performed on a 96-well format, with individual caps (MicroAmp Optical 96-well reaction plate) using BigDye Terminator chemistry (BigDye v 3.1 terminator, Applied BioSystem®, Courtaboeuf, France) on GeneAmp-9700 (Applied BioSystem®, Courtaboeuf, France). Sequencing was realized with 10 µL of EDTA 0.3 mM par well on automatic DNA analyser ABI 3700 (Applied BioSystem®, Courtaboeuf, France). For data analysis, we used SeqScape® software (Applied BioSystem®, Courtaboeuf, France).

msp-1 and msp-2 genotyping. Polymerase chain reaction genotyping was performed as described by Snounou,29 using
RESULTS

Among 381 blood samples collected from *P. falciparum*-infected individuals, only monoclonal samples (1 genotype found with *msp*-1 and *msp*-2 genotyping) with most prevalent genotypes and data from the *in vitro* isotopic drug sensitivity assay were analyzed: chloroquine (*N* = 134), monodesethylamodiaquine (*N* = 134), quinine (*N* = 135), dihydroartemisinin (*N* = 51), halofantrine (*N* = 24), mefloquine (*N* = 49), and pyrimethamine (*N* = 39). The mean age of the *P. falciparum*-infected individuals was 25 years (range, 1–80 years) and 36% were male. The geometric mean of parasitemia was 15,442 parasites/μl of blood (range 78–443,071 parasites/μl of blood).

Data from the *in vitro* isotopic drug sensitivity assay, the baseline frequency distribution of the mutant alleles of *pfcr*, *pfmdr1*, *dhfr*, and *dhps* genes and clonality of infections by *msp*-1 and *msp*-2 genotyping profiles have been previously described. Briefly, mutant alleles of the *pfcr* gene were found in two thirds of samples and the frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%). Frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%). Frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%). Frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%). Frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%). Frequencies of the deduced haplotype were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): common (18.0%).

Correlation between polymorphisms in the *pfcr* and *pfmdr1* genes and *in vitro* chloroquine susceptibility. For polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 in positions 86 and 1246 (*P* > 0.05, data not shown).

For the *pfcr* gene, we compared geometric means of IC50 values for deduced haplotypes in the *pfcr* gene and observed significant differences (*P* = 0.001) between haplotype with wild-type alleles (CMNK, *N* = 55, mean IC50 = 50 nM, CI 95% 32–68) and haplotypes with mutant-type alleles: CIET, 3 times higher (*N* = 63, mean IC50 = 151 nM, CI 95% 120–181); CMNT, 2.9 times higher (*N* = 10, mean IC50 = 143 nM, CI 95% 43–242) and CIEK, 2.7 times higher (*N* = 6, mean IC50 = 135 nM, CI 95% 18–280). Figure 1 shows scatter plots of the data.

Haplotypes with mutant-type alleles CIET, CMNT, CIEK were present respectively in 76%, in 75%, and in 55% chloroquine-resistant isolates (*P* = 0.005) and haplotype with wild-type allele CMNK in 85% chloroquine-sensitive isolates (*P* = 0.002). We estimated that deduced haplotypes CIET, CMNT, CIEK were 3.2 times, 2.9 times, and 1.2 times more frequent, respectively, in the chloroquine-resistant phenotype.

The positive predictive value (PPV) of mutant-type I74, E75, and T76 alleles for chloroquine-resistance were estimated to 54%, 20%, and 74%, respectively.

**Correlation between polymorphisms in the *pfcr* and *pfmdr1* genes and *in vitro* monodesethylamodiaquine susceptibility.** For polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 values in positions 86 and 1246 (*P* > 0.05, data not shown).

For the *pfcr* gene, we found significant increases for geometric mean of IC50 values for *in vitro* monodesethylamodiaquine susceptibility (*P* = 0.002): 2 times higher for the CIET haplotype (*N* = 62, mean IC50 = 52 nM, CI 95% 39–64) than the CMNK haplotypes (*N* = 56, mean IC50 = 26 nM, CI 95% 21–32). Figure 1 shows scatter plots of the data.

The deduced haplotypes CIET was present in 85% monodesethylamodiaquine-resistant isolates (*P* = 0.02) and CMNK haplotype in 95% monodesethylamodiaquine-sensitive isolates (*P* = 0.006). We estimated that deduced haplotypes CIET was 1.6 times more frequent in the monodesethylamodiaquine-resistant phenotype. We estimated the PPV of mutant-type I74, E75, and T76 alleles for monodesethylamodiaquine resistance as 22%, 22%, and 24%, respectively.

**Correlation between polymorphisms in the *pfcr* and *pfmdr1* genes and *in vitro* quinine susceptibility.** For polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 in positions 86 and 1246 (*P* > 0.05, data not shown).

For the *pfcr* gene, we also found a significant difference (*P* = 0.03) between the CIET (*N* = 64, mean IC50 = 213 nM, CI 95% 183–244 nM) and CMNK (*N* = 55, mean IC50 = 163 nM, CI 95% 128–159 nM) haplotypes (1.3 times higher for CIET). Figure 1 shows scatter plots of the data.

**Correlation between polymorphisms in the *pfcr* and *pfmdr1* genes and *in vitro* susceptibilities of others antimalarial drugs tested.** For polymorphisms in the *pfmdr1* and *pfcr* genes, we found no significant differences (*P* > 0.05, data not shown) for the geometric means of the IC50 values for *in vitro* dihydroartemisinin, halofantrine, and mefloquine susceptibility.
Correlation between polymorphisms in *dhfr* and *in vitro* pyrimethamine susceptibility. We found significant increases in the geometric means of the IC50 values for *in vitro* pyrimethamine susceptibility (*P* = 0.0002): 10 times higher for the mutant-type I51 allele (*N* = 27, mean IC50 = 5288 nM, CI 95% 3614–6961 nM) than the wild-type N51 allele (*N* = 12, mean IC50 = 502 nM, CI 95% 118–886 nM); 21 times higher for the mutant-type R59 allele (*N* = 31, mean IC50 = 4742 nM, CI 95% 3201–6283 nM) than the wild-type C59 allele (*N* = 8, mean IC50 = 225 nM, CI 95% 85–489 nM); 21 times higher for the mutant-type N108 allele (*N* = 30, mean IC50 = 4888 nM, CI 95% 3324–6453 nM) than the wild-type S108 allele (*N* = 9, mean IC50 = 238 nM, CI 95% 16–484 nM). We found 100% of wild-type alleles for codons 16 and 50, one sample with mutant-type 164L and one sample with mixed type 164I/L (not analyzed in this study).

For the deduced haplotypes in *dhfr* gene, the geometric means of the IC50 values for *in vitro* pyrimethamine susceptibility increased regularly with the number of mutation (*P* = 0.002): 22 times higher between ACNCN (4, mean IC50 = 299 nM, CI 95% 123–1210) and ACNCSI haplotypes (5, mean IC50 = 13.6 nM, CI 95% 2–88), 53 times higher between ACIRSI (4, mean IC50 = 720 nM, CI 95% 253–1420) and ACNCSI haplotypes, 80 times higher between ACNRNI (3, mean IC50 = 1081 nM, CI 95% 524–2229) and ACNCSI haplotypes, and 332 times higher for ACIRNI haplotype (23, mean IC50 = 4323 nM, CI 95% 3140–5951) and ACNCSI haplotypes. Figure 2 shows scatter plots of the data.

The deduced haplotypes ACIRNI, ACIRSI, ACNRNI, and ACNCNI were only present in pyrimethamine-resistant isolates (*P* = 10<sup>−5</sup>) and the deduced haplotypes ACNCSI in the pyrimethamine-sensitive isolates (*P* = 10<sup>−4</sup>).

We estimated that the mutant-type I51, R59, and N108 alleles were 27 times, 30 times, 30 times more frequent, respectively, in the pyrimethamine-resistant phenotype. We estimated the PPV of mutant-type I51 and N108 alleles for

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**Figure 1.** *In vitro* susceptibility to chloroquine (A), mono-desethylamodiaquine (B), and quinine (C) in isolates of *Plasmodium falciparum* from the Central African Republic with deduced haplotypes in the *pfcr* gene.

**Figure 2.** *In vitro* susceptibility to pyrimethamine in isolates of *Plasmodium falciparum* from the Central African Republic with the number of mutant allele in the *dhfr* gene.
pyrimethamine-resistant phenotype as 100% for each allele and as 96.8% for the mutant-type R59 allele.

**DISCUSSION**

Drug resistance is probably the greatest challenge to most malaria-control programs. Given the limited resources for other malarial-control measures, rational drug used is crucial, although economic constraints and the scarcity of drug choice hampers this. Close monitoring of the epidemiology and dynamics of drug resistance are necessary to implement measures to circumvent the problem and to allow the National Malaria Control Program to recommend the best available management of malaria.

We have previously shown a close relationship between in vivo and in vitro studies in Bangui. We used the standard 14-day WHO 2001 protocol to compare therapeutic responses in children less than 5 years of age with acute uncomplicated *Plasmodium falciparum* malaria in Bangui between February 2002 and March 2004. We found that the overall treatment failure rates with chloroquine, amodiaquine sulfadoxine-pyrimethamine, chloroquine + sulfadoxine-pyrimethamine combination, and amodiaquine + sulfadoxine-pyrimethamine combination were 40.9%, 20.0%, 22.8%, 7.2%, and 0%, respectively.25 Consistent with this were in vitro isotopic drug sensitivity assays carried out in Bangui between March and July 2004. They showed that the proportion of resistant isolates was 37% for chloroquine, 15.9% for amodiaquine, 0% for quinine, 0% for dihydroartemisinin, 1.6% for melfloquine, 3.8% for halofantrine, 4.0% for atovaquone, and 83% for pyrimethamine.26 We also determined with the same samples, the baseline frequency distribution of the mutant alleles of genes associated with resistance to chloroquine and sulfadoxine-pyrimethamine. We found mutant alleles of the *pfcrt* gene in two thirds of the samples and a high (45%) frequency of the deduced CIET *pfcrt* haplotype. The most common alleles of the *pfmdr1* gene among the field isolates of *P. falciparum* were the 86Y (21.9%) and 1246Y (18.0%) alleles. Analysis of *dhfr* gene showed that only 12 isolates carried the wild-type allele (7.2%), whereas many (57.5%) were triple mutants (51L, 59R, 108N).27 More interesting, was, for the first time in Central African Republic, the quadruple mutation (51L/59R/108/164) in 2 samples, using the PCRRFLP approach, this finding being confirmed by sequencing, subsequently. Unfortunately, IC50 values for in vitro pyrimethamine susceptibility were not available for these samples. According to Nzila and colleagues,31 this result indicated that the quadruple mutant DHFR exists in Central African Republic where SP is widely used, implying that the useful therapeutic life of the new combination such as chlorproguanil plus dapsone (Lapdap98) could be very short.

In this present study, we have shown a strong association between the *dhfr* genotype and chemosensitivity to pyrimethamine. Isolates with single or combined *dhfr* I51, R59, or N108 mutations were between 10 and 21 times less susceptible to pyrimethamine. For pyrimethamine, we found a regular increase in IC50 values with the number of *dhfr* mutations, from a 22-fold increase for a single mutant-type allele to a 332-fold increase for a triple mutant. This is consistent with previous studies correlating *dhfr* mutations and chemosensitivity in field samples from Brazil32 and Africa.33–35 We also observed a strong relationship between phenotype and geno-

- type for pyrimethamine (all except one isolate containing a mutant allele was found to be resistant), the PPVs of mutant-type I51 and N108 alleles for pyrimethamine-resistant phenotype being 100% and the PPV of mutant-type R59 being 96.8%. Therefore, we believe that PCR-RFLP analysis in the *dhfr* gene for codons 51, 59, and 108 may be a quick and reliable method being able to replace the in vitro techniques providing particularly poor results for pyrimethamine. However, associations between theses molecular markers and clinical SP treatment outcomes seem to be less straightforward. Even if several studies such as Kublin and associates20 showed that the presence together of all 5 *dhfr* and *dhps* mutations that are found in Africa were strongly associated with SP failure, recent study conducted by Francis and colleagues28 strongly suggest that geographic differences in response to antimalarial therapy in Uganda are primarily mediated by acquired immunity associated with malaria transmission intensity, rather than by parasite factors such as molecular markers of antimalarial drug resistance.

We have evaluated the correlation between *pfcrt* and *pfmdr1* mutations and in vitro responses to chloroquine, monodesethylamodiaquine, quinine, dihydroartemisinin, melfloquine, and halofantrine. We found that for haplotypes with mutant-type alleles in the *pfcrt* gene led to significant changes in the IC50 values of chloroquine, monodesethylamodiaquine, and quinine. This is consistent with our previous in vitro study,26 in which we found significant differences in the IC50 values for monodesethylamodiaquine and quinine in chloroquine-sensitive and chloroquine-resistant isolates, and in which there was a significant correlation between the in vitro responses to chloroquine and monodesethylamodiaquine (r = 0.61) and to chloroquine and quinine (r = 0.33). The cross-resistance between these drugs can be partly explained by similarities in their chemical structures.37 Among the 4 haplotypes (CMNK, CIEK, CMNT, and CIET) found in *pfcrt* gene, CMNK haplotype was clearly associated with chloroquine-resistant phenotype and sulfadoxine-pyrimethamine. We found mutant alleles of the dhfr gene in two thirds of the samples and a high (45%) frequency of the deduced CIET dhfr haplotype. The most common alleles of the pfmdr1 gene among the field isolates of P. falciparum were the 86Y (21.9%) and 1246Y (18.0%) alleles. Analysis of dhfr gene showed that only 12 isolates carried the wild-type allele (7.2%), whereas many (57.5%) were triple mutants (51L, 59R, 108N).27 More interesting, was, for the first time in Central African Republic, the quadruple mutation (51L/59R/108/164) in 2 samples, using the PCRRFLP approach, this finding being confirmed by sequencing, subsequently. Unfortunately, IC50 values for in vitro pyrimethamine susceptibility were not available for these samples. According to Nzila and colleagues,31 this result indicated that the quadruple mutant DHFR exists in Central African Republic where SP is widely used, implying that the useful therapeutic life of the new combination such as chlorproguanil plus dapsone (Lapdap98) could be very short.

In this present study, we have shown a strong association between the dhfr genotype and chemosensitivity to pyrimethamine. Isolates with single or combined dhfr I51, R59, or N108 mutations were between 10 and 21 times less susceptible to pyrimethamine. For pyrimethamine, we found a regular increase in IC50 values with the number of dhfr mutations, from a 22-fold increase for a single mutant-type allele to a 332-fold increase for a triple mutant. This is consistent with previous studies correlating dhfr mutations and chemosensitivity in field samples from Brazil32 and Africa.33–35 We also observed a strong relationship between phenotype and geno-

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Much confusion has surrounded the association of different *pfmdr1* alleles to chloroquine resistance, although transfection studies as well as carefully controlled, molecular epidemiologic studies have shown that there are strong associations between polymorphisms and antimalarial resistance.42–45
However, like many other studies,56–86 our present findings have failed to find such associations because the presence of both wild-type and mutant-type alleles in our samples were largely independent of their in vitro responses. This can be for a variety of reasons, including the inability to control for the inoculum effect,49 which can alter the in vitro sensitivity erroneously. In your opinion and according to Duraisingh and associates,13 there may be true epidemiologic differences in resistance resulting from pfmdr1 polymorphisms in different populations and differences in the strength of associations between resistance and pfmdr1 alleles could reflect the different selection histories of these populations. However, correlations between arylaminoalcohols or endoperoxide artemisinin derivatives resistance and amplification of pfmdr1 gene90,91 or new candidate transporter gene92 merit further investigation.

In conclusion, these findings suggest that analysis of the dhfr and pfcrt gene may be a reliable tool for the epidemiologic surveillance of antimalarial drug resistance in the CAR. A regular monitoring and screening for resistance markers for antifolates (such as the dhfr mutations) and for chloroquine (such as the pfcrt mutations) could act as an adjunct to in vivo trials and should be carried out both in Bangui and in the CAR, to allow the National Malaria Control Program to recommend the best available management of malaria (antimalarial drugs used as first-line treatment or intermittent preventive treatment during pregnancy).

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