

Amodiaquine Metabolism is Impaired by Common Polymorphisms in CYP2C8: Implications for Malaria Treatment in Africa

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Metabolism of the antimalarial drug amodiaquine (AQ) into its primary metabolite, *N*-desethylamodiaquine, is mediated by CYP2C8. We studied the frequency of CYP2C8 variants in 275 malaria-infected patients in Burkina Faso, the metabolism of AQ by CYP2C8 variants, and the impact of other drugs on AQ metabolism. The allele frequencies of CYP2C8*2 and CYP2C8*3 were 0.155 and 0.003, respectively. No evidence was seen for influence of CYP2C8 genotype on AQ efficacy or toxicity, but sample size limited these assessments. The variant most common in Africans, CYP2C8*2, showed defective metabolism of AQ (threefold higher K_m and sixfold lower intrinsic clearance), and CYP2C8*3 had markedly decreased activity. Considering drugs likely to be coadministered with AQ, the antiretroviral drugs efavirenz, saquinavir, lopinavir, and tipranavir were potent CYP2C8 inhibitors at clinically relevant concentrations. Variable CYP2C8 activity owing to genetic variation and drug interactions may have important clinical implications for the efficacy and toxicity of AQ.

Malaria, in particular that caused by *Plasmodium falciparum*, remains among the leading causes of morbidity and mortality in the developing world.¹ Recent estimates suggest that more than 500 million episodes of *P. falciparum* malaria occurred in 2002, leading to one to three million deaths.^{2,3} The burden of malaria is heaviest in sub-Saharan Africa, where resistance to the most commonly employed antimalarials, in particular chloroquine and sulfadoxine-pyrimethamine (PYR), is widespread. In addition, given the level of transmission in many areas, individuals may receive several short courses of antimalarial therapy every year.² To combat the emergence and spread of resistance, the World Health Organization (WHO) has recommended the use of combination antimalarial therapy for *P. falciparum* malaria. Amodiaquine (AQ), a 4-aminoquinoline similar in structure to chloroquine, is included in two of five recommended regimens, AQ + artesunate and AQ + sulfadoxine-PYR.⁴ In addition, AQ monotherapy is still widely used to treat malaria in Africa.

AQ was introduced as an antimalarial in the 1940s. In the mid-1980s, AQ administration increased largely owing to

increased prophylactic use in Western travelers. However, several reports soon emerged suggesting an unacceptable level of toxicity of AQ, in particular agranulocytosis (estimated 1:2,100 users with a fatality rate of 1:31,000)^{5,6} and hepatotoxicity (1:15,600 with numerous fatalities).^{5,7} Recommendations for chemoprophylaxis with AQ were dropped, and the WHO removed AQ from its Essential Drugs List in 1990.⁸ However, subsequent review has suggested that AQ toxicity was primarily seen in non-Africans receiving long-term chemoprophylaxis and the drug was reinstated in 1996 by the WHO as an option for treating malaria, with millions of dosages subsequently given each year.^{9,10}

The metabolism of AQ has been characterized in studies using human liver microsomes and recombinant enzymes. AQ is metabolized to its primary metabolite, *N*-desethylamodiaquine (DEAQ), by the cytochrome P450 (CYP) 2C8 enzyme,^{11,12} which accounts for 7% of the total microsomal CYP content of the liver¹³ and is estimated to carry out oxidative metabolism of at least 5% of drugs.¹⁴ Three relatively common sequence-altering variants are denoted as

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Received 10 November 2006; accepted 20 December 2006; advance online publication 14 March 2007. doi:10.1038/sj.cpt.6100122

*CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4*, with the wild-type denoted as *CYP2C8*1* (Table 1, www.imm.ki.se/CYPalleles).¹⁵ *CYP2C8*2* is most prevalent in those of African descent, whereas *CYP2C8*3* is more prevalent among Caucasians.¹⁵ Several other single nucleotide polymorphisms have been described, including coding polymorphisms and two translational stop codons in Japanese, but at much lower frequencies.^{14,16,17} Both *CYP2C8*2* and *CYP2C8*3* are defective in metabolism of the anticancer agent paclitaxel, and *CYP2C8*3* has reduced activity towards the endogenous substrate arachidonic acid.¹⁵ The aim of this study is to assess the impact of *CYP2C8* polymorphisms on AQ response and toxicity in a cohort of malaria infected patients in Burkina Faso and to examine the *in vitro* metabolism of AQ by *CYP2C8* variants. Additionally, we investigate the potential for significant *CYP2C8* inhibition by other drugs likely to be coadministered with AQ in sub-Saharan Africa.

RESULTS

Genotyping

A total of 280 subjects in the AQ monotherapy arm completed the clinical trial and had efficacy outcomes; 275 of these subjects had DNA available and were genotyped for three nonsynonymous *CYP2C8* variants (Table 2). All genotypes were in Hardy–Weinberg equilibrium. The allelic frequency for *CYP2C8*2* (805A>T) was 0.115 and 25% of the population were heterozygotes. Only five (2%) individuals were homozygotes for the *CYP2C8*2* variant. The *CYP2C8*3* allele (416G>A and 1196A>G) was much less common in this African population (allele frequency 0.004). The *CYP2C8*4* (792C>G) allele was not detected.

Table 1 Major *CYP2C8* alleles in Caucasians and Africans

Allele	Location	Nucleotide change	Amino-acid effect
<i>CYP2C8*1</i>	NA	None	None
<i>CYP2C8*2</i>	Exon 5	805A>T	I269F
<i>CYP2C8*3</i>	Exon 3, Exon 8	416G>A, 1196A>G	R139K, K399R
<i>CYP2C8*4</i>	Exon 5	792C>G	I264M

NA, not available.

Table 2 *CYP2C8* allele frequencies in this study and other populations

Allele	N	<i>CYP2C8*2</i>	<i>CYP2C8*3</i>	<i>CYP2C8*4</i>	Ref
Burkina Faso	275	0.115	0.004	Not found	This study
Zanzibar	165	0.139	0.021	0.006	¹⁸
Northern Ghana	200	0.168	Not found	Not found	¹⁹
African Americans	82	0.183	0.018	Not found	¹⁵
Portugal	164	0.012	0.198	0.064	²⁰
British Caucasians	116	0.004	0.150	0.075	²¹
Sweden	1468	Not reported	0.095	Not reported	²²
Malaysian Indians	123	0.008	0.12	Not found	²³
Japanese	360	Not found	Not found	Not found	²⁴

Association with treatment outcome and adverse events

Overall, 82.2% of participants in the AQ treatment arm of the study responded successfully to treatment, with no evidence of clinical malaria or parasitemia over 28 days of follow-up.²⁵ Efficacy outcomes did not vary between *CYP2C8*1* homozygotes and *CYP2C8*2* heterozygotes (Table 3). In addition, time to therapeutic failure did not vary between these two groups (data not shown). All five *CYP2C8*2* homozygotes responded to therapy.

Adverse events were uncommon in the AQ monotherapy arm.²⁵ There was an increase in the self-reported rate of abdominal pain in both heterozygotes and homozygotes for the variant *CYP2C8*2* genotype compared with wild-type genotype (52 vs 30%, $P<0.01$). No other associations were seen between *CYP2C8*2* genotype and specific adverse events, including nausea, vomiting, fatigue, and jaundice.

AQ metabolism by recombinant *CYP2C8* proteins

High-performance liquid chromatography (HPLC)/UV analysis for the metabolites of AQ after incubation with recombinant *CYP2C8* revealed a single metabolite. The metabolite was identified as DEAQ by comigration with a reference standard. Retention times for DEAQ, primaquine diphosphate, and AQ were 14.8, 16.9, and 18.1 min, respectively (Figure 1). Formation of DEAQ in *CYP2C8* incubations was linear with time and protein concentration. Under the assay conditions used in this study, metabolites such as N-bisDEAQ, 2-hydroxyDEAQ, or the M2 metabolite, were not seen. This is consistent with the available literature suggesting that metabolites such as M2 are formed extrahepatically and the lack of evidence that *CYP2C8* can catalyze the formation of metabolites other than desethylamodiaquine.^{11,26–28}

Formation of DEAQ by *CYP2C8*1* exhibited typical Michaelis–Menten kinetics (Figure 2). For the wild-type allele, *CYP2C8*1*, the apparent V_{max} was $0.23 \pm 0.09 \mu\text{mol}/\text{min}/\mu\text{mol}$ P450, with a K_m of $0.81 \pm 0.23 \mu\text{M}$. The corresponding intrinsic clearance (defined as V_{max}/K_m) was $0.30 \text{ l}/\text{min}/\mu\text{mol}$ P450. The *CYP2C8*2* allele had a significantly lower V_{max} of $0.16 \pm 0.06 \mu\text{mol}/\text{min}/\mu\text{mol}$ P450 ($P=0.04$) and a threefold higher K_m , $2.55 \pm 1.06 \mu\text{M}$ ($P=0.05$). The intrinsic clearance of AQ for *CYP2C8*2* was sixfold lower than that for *CYP2C8*1* (0.05 vs $0.30 \text{ l}/\text{min}/\mu\text{mol}$ P450, $P<0.01$). Metabolic activity was not sufficient to estimate

Table 3 Association of *CYP2C8*2* with treatment outcome and adverse events

Outcome	<i>CYP2C8*2</i> genotype, n (%)		
	AA (n=199) ^a	AT (n=67) ^a	TT (n=5)
ACPR	164 (82)	57 (85)	5 (100)
Recrudescence	20 (10)	6 (9)	0
New infection	15 (7.5)	4 (6)	0

ACPR, adequate clinical and parasitological response. ^aParasite genotyping results were not obtained for four specimens.

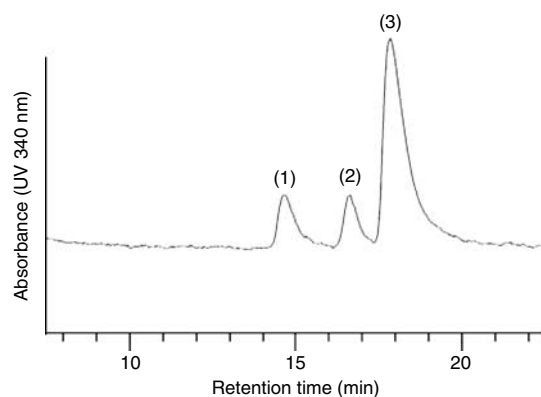


Figure 1 Chromatographic separation of (1) DEAQ, (2) primaquine internal standard, and (3) AQ. This chromatogram shows the product formation from an incubation of AQ with recombinant CYP2C8*1 protein.

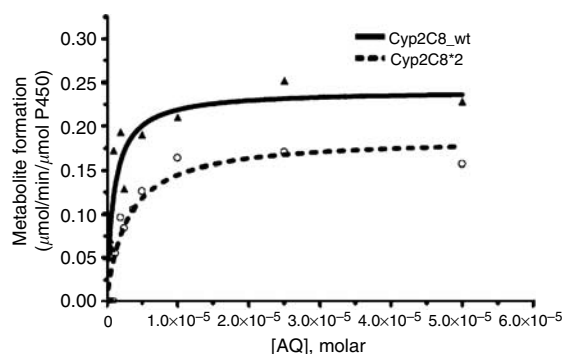


Figure 2 Plot of velocity vs AQ concentration for the formation of desethylamodiaquine by recombinant CYP2C8*1 and CYP2C8*2 proteins. Each point is the mean velocity from triplicate determinations at a given concentration and the lines were drawn using the estimated Michaelis–Menten parameters.

adequately the kinetic parameters for CYP2C8*3, as no metabolism was detectable until AQ concentrations of 15–25 μM were tested and solubility prevented measurements within the saturable range for this enzyme.

Interactions between recombinant CYP2C8 and other drugs

We studied six drugs for their impact on the AQ *N*-desethylase activity of recombinant CYP2C8 proteins (Table 4). Trimethoprim (TMP), a widely used dihydrofolate reductase inhibitor, inhibited CYP2C8 ($\text{IC}_{50} = 40.6 \pm 12.7 \mu\text{M}$). These results correlated with previous reports.^{32,34} PYR, another dihydrofolate reductase inhibitor, also inhibited CYP2C8 at similar concentrations ($\text{IC}_{50} = 45.1 \pm 12.2 \mu\text{M}$). Efavirenz, an antiretroviral non-nucleoside reverse transcriptase inhibitor that is widely used to treat human immunodeficiency virus (HIV) infection, was a potent inhibitor of CYP2C8 at clinically relevant concentrations ($\text{IC}_{50} = 4.0 \pm 2.5 \mu\text{M}$). HIV-1 protease inhibitors were also potent inhibitors of CYP2C8-mediated AQ desethylase activity: saquinavir, lopinavir, and tipranavir all inhibited the metabolism of AQ at clinically relevant concentrations.

Table 4 IC_{50} values for inhibitors of recombinant CYP2C8

Drug	IC_{50} (μM) \pm SE	Systemic concentration (μM)	
		C_{max}	C_{min}
Trimethoprim	40.6 ± 12.7	2 ^a	NA
Pyrimethamine	45.1 ± 12.2	2.4 ^b	NA
Efavirenz	4.0 ± 2.5	12.9 ^c	5.6
Nevirapine	≥ 30 ^d	21.6	14
Saquinavir	1.8 ± 0.8	5.5 ^e	0.6
Lopinavir	4.1 ± 0.6	15.6 ^f	8.8
Tipranavir	2.1 ± 0.3	78–95 ^g	36–42
Ritonavir	3.03 ± 1.14 ^h	15.5	5.1
Nelfinavir	~ 40 ⁱ	6	3.3

NA, not available; SE, standard error. IC_{50} data are means \pm SD from four experiments. Serum concentrations are from published information. C_{max} and C_{min} are the mean maximum and minimum serum levels achieved under standard dosing intervals, respectively. ^aOn the basis of trimethoprim 160 mg q.i.d. dosing in HIV-infected patients.²⁹ ^bOn the basis of pyrimethamine 750 mg single dose.³⁰ ^cOn the basis of efavirenz 600 mg q.i.d. in HIV-infected individuals (Bristol-Myers Squibb prescribing information). ^dOn the basis of nevirapine 200 mg b.i.d. in HIV-1-infected individuals³¹ and Walsky et al.³² ^eOn the basis of saquinavir 1200 mg t.i.d. (as free base) in HIV-infected individuals, and coadministered saquinavir soft gel capsule. 1,000 mg/ritonavir 100 mg b.i.d. in HIV-infected individuals (Roche prescribing information).³³ ^fOn the basis of lopinavir 400 mg/ritonavir 100 mg b.i.d. in HIV-infected individuals (Abbott prescribing information). ^gOn the basis of tipranavir 500 mg/ritonavir 200 mg b.i.d. in HIV-infected individuals (Boehringer Ingelheim Pharmaceuticals prescribing information). ^hOn the basis of ritonavir 600 mg b.i.d. in healthy and HIV-infected individuals (Abbott prescribing information) and Walsky et al.³² ⁱOn the basis of nelfinavir mesylate 1250 mg b.i.d. in HIV-infected individuals; C_{min} was determined before morning dosage (Agouron prescribing information) and Walsky et al.³²

DISCUSSION

Few studies are available on the impact of genetic variation on the metabolism of commonly used antimalarial drugs. We describe the influence of CYP2C8 polymorphisms on the metabolism of AQ. In our study population in Burkina Faso, the variant allele *CYP2C8*2* was common, with a prevalence of 11.5% and the variant most prevalent in Caucasians, *CYP2C8*3*, was rare.^{20,21} These data correlate well with other published allelic frequencies from West Africa (Table 2). Compared with the wild-type enzyme, CYP2C8*2 showed a threefold higher K_m and sixfold lower intrinsic clearance for AQ. These results are consistent with but of greater magnitude than the twofold higher K_m of recombinant CYP2C8*2 for paclitaxel and the twofold increase in intrinsic clearance for that substrate.¹⁵ The decreased AQ desethylase activity of the CYP2C8*3 variant was more profound than that of the CYP2C8*2 variant, suggesting that effects of reduced metabolism of AQ will be most pronounced in CYP2C8*3 carriers. Indeed, no appreciable metabolism by CYP2C8*3 was detectable until AQ substrate concentrations nearly 15–20-fold higher than the K_m of the wild-type enzyme were tested. CYP2C8*3 also had extremely low turnover for paclitaxel (6% of that of wild-type CYP2C8*1) and a threefold lower turnover number for arachidonic acid. *In vivo* studies have shown a 4.5-fold increase in the half-life of (*R*)-ibuprofen in subjects homozygous for the CYP2C8*3

allele compared with the CYP2C9*1 allele.³⁵ Our results for AQ metabolism by CYP2C8 variants and the reported findings for paclitaxel, arachidonic acid, and ibuprofen illustrate that the magnitude of the effect of these polymorphisms depends on the substrate and the relationship of substrate concentration to K_m .

AQ is predominantly metabolized into a single major metabolite, DEAQ.^{11,12} Several other metabolites have been described, but were not identified in our studies (Figure 3). Metabolism into DEAQ occurs rapidly, with no AQ detectable within a few hours of oral intake (terminal half-life, 5.2 h).^{36,37} In contrast, DEAQ is present in the blood for an extended period after therapy, with a terminal half-life of 9–18 days and wide interindividual variability in plasma levels.^{36,38} Both AQ and DEAQ have antimalarial activity, but AQ is up to threefold more potent.³⁹ Nonetheless, owing to its much higher concentrations, DEAQ is considered the major active component.³⁹ Given our findings of impaired conversion of AQ into DEAQ by the CYP2C8*2 and CYP2C8*3 variants, it would be predicted that for both variants AQ and DEAQ, concentration–time profiles would be significantly altered, possibly contributing to the observed pharmacokinetic variability.³⁸ However, it is not possible that such alterations in AQ metabolism would affect therapeutic efficacy, as both the parent drug and metabolite are active. Indeed, in our study no impact on antimalarial efficacy was demonstrated for the common CYP2C8*2 variant.

Of possibly greater importance than impacts on therapeutic efficacy are potential effects of CYP2C8 variants on

AQ toxicity. As noted above, long-term usage of AQ for malaria chemoprophylaxis led to important risks of blood dyscrasias and hepatic disorders.^{6,40,41} Mechanisms of AQ toxicity are not known, but both AQ and DEAQ led to inhibitory effects on bone marrow progenitor cells and neutrophil function *in vitro*.^{42–46} Additionally, several lines of evidence suggest that AQ toxicity is mediated through the production of an immunologically reactive quinoneimine,^{47,48} a finding supported by the demonstration of anti-AQ antibodies in individuals with AQ-associated toxicity.^{49–51} Importantly, DEAQ is less readily activated to immunologically reactive compounds compared with AQ.^{21,52,53} One would thus expect that individuals with impaired metabolism of AQ into DEAQ may be at increased risk of toxicity because of higher levels of AQ. We propose that the severe toxicity seen with AQ usage as a chemoprophylactic may have been influenced by a relatively high prevalence of the functionally defective CYP2C8*3 variant in Caucasians (15–20%). Although the enzymatic defect with the CYP2C8*2 allele prevalent in Africa (15–18% frequency) is less profound than that for CYP2C8*3, individuals with the variant gene might experience decreased tolerability and increased toxicity with AQ. It is reassuring that our study did not identify common toxicities or diminished tolerability for AQ in CYP2C8*2 variants. Nonetheless, as AQ is now widely used, with individuals probably receiving repeated courses of therapy, careful monitoring of larger samples for adverse events related to AQ and for associations with the CYP2C8*2 genotype are warranted.

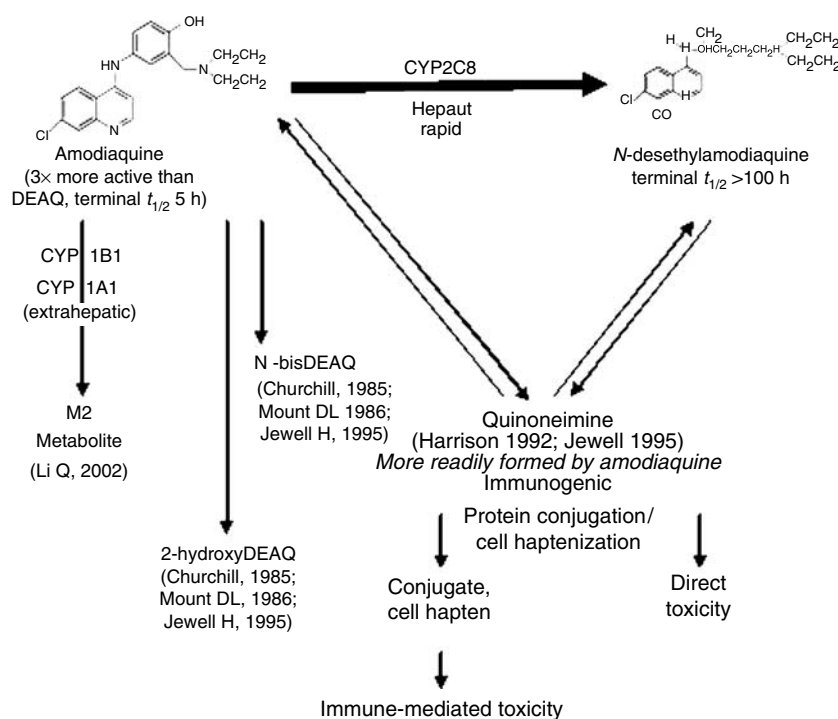


Figure 3 Metabolic pathway for AQ showing the formation of the active DEAQ, other minor metabolites, and the reactive quinoneimine implicated in immunotoxicity of AQ.

We evaluated the impact of several drugs on CYP2C8-mediated AQ metabolism. TMP is a component of TMP-sulfamethoxazole, which is widely used to prevent secondary infections in HIV-infected individuals and recommended for daily usage in all HIV-1-infected Africans by some authorities.⁵⁴ The related dihydrofolate reductase inhibitor, PYR, is coformulated with sulfadoxine and commonly administered with AQ to treat malaria.⁵⁵ In our study, both TMP and PYR inhibited CYP2C8, but only at concentrations not achieved by standard dosing of these drugs. Of particular interest for interactions with AQ are antiretroviral drugs that are increasingly available to treat the more than 25 million HIV-infected individuals in Africa.⁵⁶ Considering non-nucleoside reverse transcriptase inhibitors, nevirapine did not appreciably inhibit CYP2C8,²⁸ but efavirenz was a potent inhibitor, with an IC_{50} below the mean minimum serum levels achieved under standard dosing intervals (C_{min}) achieved with standard dosing. Considering antiretroviral protease inhibitors, previous studies showed that ritonavir (at high doses), but not nelfinavir, were potent CYP2C8 inhibitors.²⁸ In our study, saquinavir, lopinavir, and tipranavir were potent CYP2C8 inhibitors at clinically relevant concentrations. In particular, tipranavir had an IC_{50} against CYP2C8 approximately 20-fold below the minimum serum concentration achieved during standard dosing. Given the tremendous burden of HIV-malaria coinfection in Africa, such drug interactions are an important concern.⁵⁶ In addition, the inhibition of CYP2C8 by efavirenz, saquinavir, lopinavir, and tipranavir has implications not only for AQ administration but also for other substrates of CYP2C8 such as paclitaxel, thiazolidinediones, repaglinide, amiodarone, arachidonic acid, loperamide, and morphine.^{14,57} It will be of interest to examine the potential impact of antiretroviral-antimalarial interactions in clinical settings.

We have described a more significant impact of a CYP2C8 variant on the metabolism of AQ than previously described for any drug. These findings may explain, at least in part, AQ-associated toxicity seen with long-term chemoprophylactic usage in primarily Caucasian populations and they suggest that differences in AQ efficacy and toxicity may also be associated with different CYP2C8 genotypes in Africans. With the high prevalence of the CYP2C8*2 variant in Africa, and with increasing use of multiple drugs that may affect CYP2C8-mediated metabolism, additional study of the metabolism of AQ and careful monitoring for AQ-associated toxicity are warranted.

METHODS

Chemicals. Amodiaquine dihydrochloride (AQ), quinine, PYR, and dilauroylphosphatidylcholine were obtained from Sigma-Aldrich (St Louis, MO). Primaquine diphosphate and TMP were obtained from MP Biomedicals (Solon, OH). The antiretrovirals saquinavir (as free base), lopinavir, tipranavir, and efavirenz were obtained through the AIDS Reference and Reagent Program, Division of AIDS, NIAID, NIH. DEAQ, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from BD Biosciences Discovery

Labware (San Jose, CA). HPLC-grade acetonitrile was purchased from Fischer Scientific (Hampton, NH). Recombinant rat CYP reductase was obtained from Fengyun Xu (University of California, San Francisco, CA).⁵⁸

Subjects and clinical study. Details of the clinical study have been published.²⁵ Briefly, residents of Bobo-Dioulasso, Burkina Faso more than 6 months of age with uncomplicated falciparum malaria were randomized to receive sulfadoxine-PYR, AQ, or AQ plus sulfadoxine-PYR in 2004. For this substudy, only samples from patients treated with AQ monotherapy were analyzed. Patients were followed for 28 days and treatment outcomes were classified according to WHO guidelines with parasite genotyping performed to distinguish true failures (recrudescences) from new infections.⁴ At each follow-up visit study, clinicians assessed patients for adverse events, defined as any untoward medical occurrence, following International Conference on Harmonization guidelines.²⁵ The study was approved by the institutional review boards of the University of California, San Francisco and the Centre Muraz, Bobo-Dioulasso, Burkina Faso. All research subjects or their parents or guardians approved the use of clinical specimens for genetic testing.

CYP2C8 genotyping and sequencing. For genetic analysis, DNA was extracted from filter paper with chelex. Genotyping for CYP2C8*2, *3, and *4 variants was performed using predesigned primers and probes for the TaqMan 5' nuclease allelic discrimination assay on an ABI 7500 real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA). Variant alleles are listed in **Table 1**. Reactions were carried out with the following protocol: 95°C for 10 min, then 50 cycles at 92°C for 15 s and 60°C for 90 s. For confirmation of TaqMan results, random samples were amplified and sequenced. Polymerase chain reaction primers for sequencing were as described¹⁵ and their products were purified with ExoSAP-IT before direct sequencing (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Metabolism of AQ by recombinant CYP2C8. Recombinant wild-type and variant CYP2C8 allelic proteins were expressed in *Escherichia coli* and partially purified as described previously in the laboratory of author JAG (National Institute of Environmental Health Sciences, NC). To study enzyme activities, recombinant CYP2C8 proteins (5 pmol) and rat NADPH-CYP reductase (4 pmol/pmol P450) were reconstituted with dilauroylphosphatidylcholine (3 μ g/10 pmol P450) and incubated at room temperature for 3 min. The reconstituted enzymes were then preincubated in 0.1 M KPO_4 buffer, pH 7.4, containing AQ substrate for 5 min at 37°C. Reactions were initiated by the addition of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase in a final volume of 250 μ l, incubated at 37°C for 15 min, and terminated with the addition of 125 μ l ice-cold acetonitrile and 1 μ M primaquine internal standard. Samples were centrifuged at 14,000 g for 10 min, and supernatant was analyzed by HPLC. To determine enzyme kinetics, AQ was studied at eight different concentrations ranging from 0 to 100 μ M. All solvent concentrations were maintained at <0.1%.

Inhibition of AQ metabolism by selected antibiotics and antiretrovirals.

Supernatants from baculovirus-infected cells expressing human CYP2C8 were from BD Biosciences. CYP2C8 (2.5 pmol) was preincubated with inhibitor (TMP, PYR, saquinavir, lopinavir, tipranavir, or efavirenz) and buffer (0.1 M KPO_4 , pH 7.4) at 37°C for 5 min. AQ substrate was added (1 μ M, approximately the enzyme K_m) and incubated for an additional 5 min; the reaction was initiated by the addition of $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase to a final reaction volume of 250 μ l and incubated at 37°C for 15 min. Reactions were terminated

and extracted as described above, with the exception that 1 μM quinine was used as an internal standard. Typically, IC_{50} determinations were performed in triplicate at seven inhibitor concentrations, ranging from 0 to 100 μM . Inhibitor concentrations were adjusted as needed to adequately span the IC_{50} .

Analysis of AQ and metabolites. AQ and its major metabolites were detected and quantified using HPLC with UV detection. The HPLC system consisted of an Agilent 1100 Series System with an HP G1311A quaternary pump, an HP G1322A vacuum degasser, an HP G1314A UV/Vis detector, and an HP G1313A automated liquid sampler. A reverse-phase Vydac C_{18} column (4.6 \times 250 mm, 10 μM particle size) was used for analyte separation. The mobile phase consisted of water with 0.1% trifluoroacetic acid (A) and 95% acetonitrile with 0.08% trifluoroacetic acid (B). The gradient was initiated and maintained at 15% B for 5 min, followed by a linear gradient to 19% B over 20 min. Chromatography was carried out at a flow rate of 1.0 ml/min and effluent was monitored at 340 nm.

Statistical analysis. All data points represent the means of triplicate determinations. K_m , V_{max} , and IC_{50} data were determined by nonlinear regression analysis using Prism 4.0 (GraphPad software). Kinetic data were analyzed using a paired *t*-test with two-tailed significance value. Statistical associations between alleles and treatment outcome or adverse events were assessed by χ^2 test.

ACKNOWLEDGMENTS

We thank the clinical study teams and technicians in the dispensaries of Colsama, Sarlafoa, and Ouezzin-Ville and the study participants and their parents/guardians. We also thank members of the Kroetz lab for their assistance. Financial support for this work was provided by the National Institutes of Health (NIH) (5K23AI060681 and GM61390) and in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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- Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T. & Murray, C.J. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* **367**, 1747–1757 (2006).
- Breman, J.G., Alilio, M.S. & Mills, A. Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am. J. Trop. Med. Hyg.* **71**, 1–15 (2004).
- Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y. & Hay, S.I. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* **434**, 214–217 (2005).
- World Health Organization. *Guidelines for the Treatment of Malaria* (WHO Press, Geneva, Switzerland, 2006).
- Phillips-Howard, P.A. & West, L.J. Serious adverse drug reactions to pyrimethamine-sulphadoxine, pyrimethamine-dapsone and to amodiaquine in Britain. *J. R. Soc. Med.* **83**, 82–85 (1990).
- Hatton, C.S. *et al.* Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. *Lancet* **1**, 411–414 (1986).
- Raymond, J.M., Dumas, F., Baldit, C., Couzigou, P., Beraud, C. & Amouretti, M. Fatal acute hepatitis due to amodiaquine. *J. Clin. Gastroenterol.* **11**, 602–603 (1989).
- Centers for Disease Control. Agranulocytosis associated with the use of amodiaquine for malaria prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **35**, 165–166 (1986).
- Olliaro, P. *et al.* Systematic review of amodiaquine treatment in uncomplicated malaria. *Lancet* **348**, 1196–1201 (1996).
- Olliaro, P. & Mussano, P. Amodiaquine for treating malaria. *Cochrane Database Syst. Rev.* **2**, CD000016 (2003).
- Li, X.Q., Bjorkman, A., Andersson, T.B., Ridderstrom, M. & Masimirembwa, C.M. Amodiaquine clearance and its metabolism to *N*-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. *J. Pharmacol. Exp. Ther.* **300**, 399–407 (2002).
- Li, X.Q., Bjorkman, A., Andersson, T.B., Gustafsson, L.L. & Masimirembwa, C.M. Identification of human cytochrome P(450)s that metabolise anti-parasitic drugs and predictions of *in vivo* drug hepatic clearance from *in vitro* data. *Eur. J. Clin. Pharmacol.* **59**, 429–442 (2003).
- Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. & Guengerich, F.P. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* **270**, 414–423 (1994).
- Totah, R.A. & Rettie, A.E. Cytochrome P450 2C8: substrates, inhibitors, pharmacogenetics, and clinical relevance. *Clin. Pharmacol. Ther.* **77**, 341–352 (2005).
- Dai, D. *et al.* Polymorphisms in human CYP2C8 decrease metabolism of the anticancer drug paclitaxel and arachidonic acid. *Pharmacogenetics* **11**, 597–607 (2001).
- Hichiya, H. *et al.* Functional characterization of five novel CYP2C8 variants, G171S, R186X, R186G, K247R, and K383N, found in a Japanese population. *Drug. Metab. Dispos.* **33**, 630–636 (2005).
- Soyama, A. *et al.* Five novel single nucleotide polymorphisms in the CYP2C8 gene, one of which induces a frame-shift. *Drug Metab. Pharmacokinet.* **17**, 374–377 (2002).
- Cavaco, I. *et al.* CYP2C8 polymorphism frequencies among malaria patients in Zanzibar. *Eur. J. Clin. Pharmacol.* **61**, 15–18 (2005).
- Rower, S. *et al.* Short communication: high prevalence of the cytochrome P450 2C8*2 mutation in Northern Ghana. *Trop. Med. Int. Health* **10**, 1271–1273 (2005).
- Cavaco, I., Piedade, R., Gil, J.P. & Ribeiro, V. CYP2C8 polymorphism among the Portuguese. *Clin. Chem. Lab. Med.* **44**, 168–170 (2006).
- Bahadur, N. *et al.* CYP2C8 polymorphisms in Caucasians and their relationship with paclitaxel 6 α -hydroxylase activity in human liver microsomes. *Biochem. Pharmacol.* **64**, 1579–1589 (2002).
- Yasar, U. *et al.* Linkage between the CYP2C8 and CYP2C9 genetic polymorphisms. *Biochem. Biophys. Res. Commun.* **299**, 25–28 (2002).
- Muthiah, Y.D., Lee, W.L., Teh, L.K., Ong, C.E. & Ismail, R. Genetic polymorphism of CYP2C8 in three Malaysian ethnics: CYP2C8*2 and CYP2C8*3 are found in Malaysian Indians. *J. Clin. Pharm. Ther.* **30**, 487–490 (2005).
- Nakajima, M. *et al.* Genetic polymorphisms of CYP2C8 in Japanese population. *Drug Metab. Dispos.* **31**, 687–690 (2003).
- Zongo, I. *et al.* Amodiaquine, sulfadoxine-pyrimethamine, and combination therapy for uncomplicated falciparum malaria: a randomized controlled trial from Burkina Faso. *Am. J. Trop. Med. Hyg.* **73**, 826–832 (2005).
- Churchill, F.C., Mount, D.L., Patchen, L.C. & Bjorkman, A. Isolation, characterization and standardization of a major metabolite of amodiaquine by chromatographic and spectroscopic methods. *J. Chromatogr.* **377**, 307–318 (1986).
- Mount, D.L., Patchen, L.C., Nguyen-Dinh, P., Barber, A.M., Schwartz, I.K. & Churchill, F.C. Sensitive analysis of blood for amodiaquine and three metabolites by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **383**, 375–386 (1986).
- Jewell, H., Maggs, J.L., Harrison, A.C., O'Neill, P.M., Ruscoe, J.E. & Park, B.K. Role of hepatic metabolism in the bioactivation and detoxication of amodiaquine. *Xenobiotica* **25**, 199–217 (1995).
- Ribera, E. *et al.* Rifampin reduces concentrations of trimethoprim and sulfamethoxazole in serum in human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **45**, 3238–3241 (2001).
- Bustos, D.G. *et al.* Pharmacokinetics of sequential and simultaneous treatment with the combination chloroquine and sulfadoxine-pyrimethamine in acute uncomplicated *Plasmodium falciparum* malaria in the Philippines. *Trop. Med. Int. Health* **7**, 584–591 (2002).
- van Heeswijk, R.P. *et al.* The steady-state pharmacokinetics of nevirapine during once daily and twice daily dosing in HIV-1-infected individuals. *AIDS* **14**, F77–F82 (2000).
- Walsky, R.L., Gaman, E.A. & Obach, R.S. Examination of 209 drugs for inhibition of cytochrome P450 2C8. *J. Clin. Pharmacol.* **45**, 68–78 (2005).
- Veldkamp, A.I. *et al.* Steady-state pharmacokinetics of twice-daily dosing of saquinavir plus ritonavir in HIV-1-infected individuals. *J. Acquir. Immune. Defic. Syndr.* **27**, 344–349 (2001).

34. Wen, X., Wang, J.S., Backman, J.T., Laitila, J. & Neuvonen, P.J. Trimethoprim and sulfamethoxazole are selective inhibitors of CYP2C8 and CYP2C9, respectively. *Drug Metab. Dispos.* **30**, 631–635 (2002).
35. Martinez, C., Garcia-Martin, E., Blanco, G., Gamito, F.J., Ladero, J.M. & Agundez, J.A. The effect of the cytochrome P450 CYP2C8 polymorphism on the disposition of (R)-ibuprofen enantiomer in healthy subjects. *Br. J. Clin. Pharmacol.* **59**, 62–69 (2005).
36. Pussard, E., Verdier, F., Faurisson, F., Scherrmann, J.M., Le Bras, J. & Blayo, M.C. Disposition of monodesethylamodiaquine after a single oral dose of amodiaquine and three regimens for prophylaxis against *Plasmodium falciparum* malaria. *Eur. J. Clin. Pharmacol.* **33**, 409–414 (1987).
37. Winstanley, P., Edwards, G., Orme, M. & Breckenridge, A. The disposition of amodiaquine in man after oral administration. *Br. J. Clin. Pharmacol.* **23**, 1–7 (1987).
38. White, N.J. *et al.* Pharmacokinetics of intravenous amodiaquine. *Br. J. Clin. Pharmacol.* **23**, 127–135 (1987).
39. Churchill, F.C., Patchen, L.C., Campbell, C.C., Schwartz, I.K., Nguyen-Dinh, P. & Dickinson, C.M. Amodiaquine as a prodrug: importance of metabolite(s) in the antimalarial effect of amodiaquine in humans. *Life Sci.* **36**, 53–62 (1985).
40. Larrey, D. *et al.* Amodiaquine-induced hepatitis. A report of seven cases. *Ann. Intern. Med.* **104**, 801–803 (1986).
41. Neftel, K.A., Woodtly, W., Schmid, M., Frick, P.G. & Fehr, J. Amodiaquine induced agranulocytosis and liver damage. *Br. Med. J. (Clin. Res. Ed.)* **292**, 721–723 (1986).
42. Naisbitt, D.J., Ruscoe, J.E., Williams, D., O'Neill, P.M., Pirmohamed, M. & Park, B.K. Disposition of amodiaquine and related antimalarial agents in human neutrophils: implications for drug design. *J. Pharmacol. Exp. Ther.* **280**, 884–893 (1997).
43. Winstanley, P.A., Coleman, J.W., Maggs, J.L., Breckenridge, A.M. & Park, B.K. The toxicity of amodiaquine and its principal metabolites towards mononuclear leucocytes and granulocyte/monocyte colony forming units. *Br. J. Clin. Pharmacol.* **29**, 479–485 (1990).
44. Labro, M.T. & el Benna, J. Effect of monodesethyl amodiaquine on human polymorphonuclear neutrophil functions *in vitro*. *Antimicrob. Agents Chemother.* **35**, 824–830 (1991).
45. Aymard, J.P., Wioland, C., Ferry, R., Netter, P. & Streiff, F. The *in vitro* effect of amodiaquine on bone marrow granulocyte-macrophage progenitor cells from normal subjects. *Fundam. Clin. Pharmacol.* **6**, 1–4 (1992).
46. Rhodes, E.G., Ball, J. & Franklin, I.M. Amodiaquine induced agranulocytosis: inhibition of colony growth in bone marrow by antimalarial agents. *Br. Med. J. (Clin. Res. Ed.)* **292**, 717–718 (1986).
47. Nelson, S.D. Mechanisms of the formation and disposition of reactive metabolites that can cause acute liver injury. *Drug Metab. Rev.* **27**, 147–177 (1995).
48. Maggs, J.L., Kitteringham, N.R., Breckenridge, A.M. & Park, B.K. Autoxidative formation of a chemically reactive intermediate from amodiaquine, a myelotoxin and hepatotoxin in man. *Biochem. Pharmacol.* **36**, 2061–2062 (1987).
49. Christie, G., Breckenridge, A.M. & Park, B.K. Drug-protein conjugates—XVIII. Detection of antibodies towards the antimalarial amodiaquine and its quinone imine metabolite in man and the rat. *Biochem. Pharmacol.* **38**, 1451–1458 (1989).
50. Clarke, J.B., Neftel, K., Kitteringham, N.R. & Park, B.K. Detection of antidrug IgG antibodies in patients with adverse drug reactions to amodiaquine. *Int. Arch. Allergy Appl. Immunol.* **95**, 369–375 (1991).
51. Rouveix, B., Coulombel, L., Aymard, J.P., Chau, F. & Abel, L. Amodiaquine-induced immune agranulocytosis. *Br. J. Haematol.* **71**, 7–11 (1989).
52. Tingle, M.D., Jewell, H., Maggs, J.L., O'Neill, P.M. & Park, B.K. The bioactivation of amodiaquine by human polymorphonuclear leucocytes *in vitro*: chemical mechanisms and the effects of fluorine substitution. *Biochem. Pharmacol.* **50**, 1113–1119 (1995).
53. Harrison, A.C., Kitteringham, N.R., Clarke, J.B. & Park, B.K. The mechanism of bioactivation and antigen formation of amodiaquine in the rat. *Biochem. Pharmacol.* **43**, 1421–1430 (1992).
54. Mermin, J. *et al.* Cotrimoxazole prophylaxis by HIV-infected persons in Uganda reduces morbidity and mortality among HIV-uninfected family members. *AIDS* **19**, 1035–1042 (2005).
55. Staedke, S.G., Mpimbaza, A., Kamya, M.R., Nzarubara, B.K., Dorsey, G. & Rosenthal, P.J. Combination treatments for uncomplicated falciparum malaria in Kampala, Uganda: randomised clinical trial. *Lancet* **364**, 1950–1957 (2004).
56. Merson, M.H. The HIV-AIDS pandemic at 25—the global response. *New Engl. J. Med.* **354**, 2414–2417 (2006).
57. Projean, D., Morin, P.E., Tu, T.M. & Ducharme, J. Identification of CYP3A4 and CYP2C8 as the major cytochrome P450s responsible for morphine *N*-demethylation in human liver microsomes. *Xenobiotica* **33**, 841–854 (2003).
58. Xu, F., Falck, J.R., Ortiz de Montellano, P.R. & Kroetz, D.L. Catalytic activity and isoform-specific inhibition of rat cytochrome p450 4F enzymes. *J. Pharmacol. Exp. Ther.* **308**, 887–895 (2004).