Determinants of Primaquine and Carboxyprimaquine Exposures in Children and Adults with *Plasmodium vivax* Malaria

Cindy S. Chu,^{a,b} James A. Watson,^{b,c} Aung Pyae Phyto,^a Htun Htun Win,^a Widi Yotyingaphiram,^a Suradet Thinraow,^a Nay Lin Soe,^a Aye Aye Aung,^a Pornpimon Wilaisrisak,^a Kanokpich Kraft,^d Mallika Imwong,^d Warunee Hanpithakpong,^c Daniel Blessborn,^{b,c} Joel Tarning,^{b,c} Stéphane Proux,^a Clare Ling,^{a,b} François H. Nosten,^{a,b} Nicholas J. White^{b,c}

^aShoklo Malaria Research Unit, Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand

^bCentre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

^cMahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

^dDepartment of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

ABSTRACT Primaquine is the only widely available drug for radical cure of *Plasmodium vivax* malaria. There is uncertainty whether the pharmacokinetic properties of primaquine are altered significantly in childhood or not. Patients with uncomplicated *P. vivax* malaria and with normal glucose-6-phosphate dehydrogenase were randomized to receive either chloroquine (25 mg base/kg of body weight) or dihydroartemisinin-piperaquine (dihydroartemisinin at 7 mg/kg and piperaquine at 55 mg/kg) plus primaquine, given either as 0.5 mg base/kg/day for 14 days or 1 mg/kg/day for 7 days. Predose day 7 venous plasma concentrations of chloroquine, desethylchloroquine, piperaquine, primaquine, and carboxyprimaquine were measured. Methemoglobin levels were measured at frequent intervals. Day 7 primaquine and carboxyprimaquine concentrations were available for 641 patients. After adjustment for the milligram-per-kilogram primaquine daily dose, day of sampling, partner drug, and fever clearance, there was a significant nonlinear relationship between age and trough primaquine and carboxyprimaquine concentrations and daily methemoglobin levels. Compared to adults 30 years of age, children 5 years of age had trough primaquine concentrations that were 0.53 (95% confidence interval [CI], 0.39 to 0.73)-fold lower, trough carboxyprimaquine concentrations that were 0.45 (95% CI, 0.35 to 0.55)-fold lower, and day 7 methemoglobin levels that were 0.87 (95% CI, 0.58 to 1.27)-fold lower. Increasing plasma concentrations of piperaquine and chloroquine and poor metabolizer CYP 2D6 alleles were associated with higher day 7 primaquine and carboxyprimaquine plasma concentrations. Higher blood methemoglobin concentrations were associated with a lower risk of recurrence. Young children have lower primaquine and carboxyprimaquine exposures and lower levels of methemoglobinemia than adults. Young children may need higher weight-adjusted primaquine doses than adults. (This study has been registered at ClinicalTrials.gov under identifier NCT01640574.)

KEYWORDS *Plasmodium vivax*, primaquine, pharmacokinetics, relapse, chloroquine, dihydroartemisinin-piperaquine, radical cure

Primaquine is used for radical cure of *Plasmodium vivax* and *Plasmodium ovale* malaria, for antimalarial chemoprophylaxis, and as a single-dose gametocytocide in *Plasmodium falciparum* malaria (1). Although the slowly eliminated 8-aminoquinoline tafenoquine has been registered in a few countries for radical cure or chemoprophylaxis in adults and pamaquine and quinocide were once used, primaquine is by far the most widely deployed drug in this class. It is generally agreed that the dosing of antimalarial chemotherapy should be designed to provide equivalent exposures to the active antimalarial across the age range (1). There is uncertainty whether or not children have lower exposures to primaquine and

Citation Chu CS, Watson JA, Phyto AP, Win HH, Yotyingaphiram W, Thinraow S, Soe NL, Aung AA, Wilaisrisak P, Kraft K, Imwong M, Hanpithakpong W, Blessborn D, Tarning J, Proux S, Ling C, Nosten FH, White NJ. 2021. Determinants of primaquine and carboxyprimaquine exposures in children and adults with *Plasmodium vivax* malaria. Antimicrob Agents Chemother 65:e01302-21. <https://doi.org/10.1128/AAC.01302-21>.

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to James A. Watson, james@tropmedres.ac.

Received 29 June 2021

Returned for modification 20 July 2021

Accepted 11 August 2021

Accepted manuscript posted online 16 August 2021

Published 18 October 2021

its bioactive metabolites than adults for the same weight-adjusted doses and thus whether they should receive higher doses than currently recommended (2–5). Primaquine is combined with either an artemisinin combination treatment (ACT) or chloroquine to prevent relapse of vivax or ovale malaria (radical cure) (1). Primaquine is a prodrug requiring metabolism to bioactive metabolites mainly via the liver cytochrome P450 isoenzyme CYP 2D6 (6). The prevalence of intermediate and nonfunctioning CYP 2D6 alleles is over 50% in some populations, particularly in Asians (7–9). Decreased CYP 2D6 function, resulting from intermediate or poor-metabolizer genotypes, potentially lowers radical-cure efficacy (10, 11).

Chloroquine has been the first-line treatment for the blood stages of *P. vivax* malaria for over 70 years (1). High-grade resistance in *P. vivax* remains confined to Oceania and Indonesia (12), but in the last decade, low-grade chloroquine resistance, made manifest by earlier appearances of relapses, has been reported increasingly in other locations. Piperaquine (a slowly eliminated bisquinoline), combined with dihydroartemisinin (DHA), is a well-tolerated and highly efficacious ACT which provides an excellent alternative blood-stage treatment for *P. vivax* malaria (13). An open-label two-way randomized controlled trial, conducted on the Thailand-Myanmar border, showed previously that a 7-day high-dose primaquine regimen (1 mg/kg of body weight/day), combined with either chloroquine or dihydroartemisinin-piperaquine, was noninferior to the standard 14-day (0.5-mg/kg/day) regimen (14). This large study allowed investigation of the pharmacokinetic properties of primaquine in adults and children, characterization of the determinants of drug exposures, and correlation with therapeutic outcomes.

RESULTS

Between February 2012 and July 2014, 680 patients with uncomplicated acute *P. vivax* malaria were enrolled. In total, 641 patients had at least one day 7 plasma primaquine and carboxyprimaquine concentration measured, either during the enrollment episode or during a recurrent *P. vivax* episode (there were a total of 720 episodes with day 7 concentrations, including patients with multiple episodes). For 692 of these day 7 concentrations, the corresponding plasma chloroquine or piperaquine concentration was also available (measured from the same blood sample). We excluded 41 drug measurements considered not to be trough levels (see Materials and Methods) resulting in a total of 679 day 7 measurements included in the analysis.

In total, 71 of the patients with measured primaquine and carboxyprimaquine concentrations were 6 to ≤ 10 years of age (median weight, 20 kg; range, 13 to 30 kg), and 34 were ≤ 5 years of age (median weight, 14 kg; range, 8 to 20 kg) (Table 1). Three patients were less than 3 years old, and the youngest was 1 year 6 months old. Fever and parasite clearance times were similar across all age groups. The proportion of patients with microscopy-detected gametocytemia was higher in the older age groups (>10 years).

Effect of age. After adjustment for the daily milligram-per-kilogram primaquine dose, the number of days of dosing, the fever clearance times, and the partner drug (piperaquine versus chloroquine), there was a significant nonlinear association between age and day 7 primaquine trough concentration ($P < 10^{-16}$ for the smooth spline term fit to age) and day 7 carboxyprimaquine trough concentration ($P < 10^{-16}$ for the smooth spline term fit to age) (Fig. 1). For both primaquine and carboxyprimaquine, increasing age correlated with higher trough levels. The strongest effect was seen for the carboxyprimaquine trough levels. Thus, the ratio of carboxyprimaquine to primaquine was also associated with age, with an approximate doubling when we compared patients aged ≤ 5 years with patients 30 years or older (Fig. 1).

Effect of the partner drug. Our previously reported healthy volunteer studies showed that coadministration of piperaquine and chloroquine increased exposures to both primaquine and carboxyprimaquine (15, 16). In this study of patients with vivax malaria, a 10-fold increase in the day 7 plasma chloroquine concentrations was associated with a 2.5-fold increase in day 7 plasma primaquine concentrations (95%

TABLE 1 Patient characteristics at enrollment by age group

Parameter ^a	Values for age group ^b :			
	≤5 yrs (n = 34)	6–10 yrs (n = 71)	11–15 yrs (n = 118)	≥16 yrs (n = 418)
No. (%) of patients given chloroquine + primaquine-7	8 (5)	22 (13)	29 (18)	104 (64)
No. (%) of patients given chloroquine + primaquine-14	10 (6)	18 (11)	29 (18)	103 (65)
No. (%) of patients given DHAP + primaquine-7	7 (4)	15 (10)	37 (24)	98 (62)
No. (%) of patients given DHAP + primaquine-14	9 (6)	16 (10)	23 (14)	113 (70)
Median age in yrs (IQR, range)	4 (3.2–5, 1.5–5)	8 (6–10, 5.1–10)	13 (12–14, 11–15)	28 (20–39, 16–63)
Median wt in kg (IQR, range)	14 (12–16, 8–20)	20 (16–23, 13–30)	35 (29–43, 21–57)	52 (47–56, 31–88)
Median body mass index in kg/m ² (IQR, range)	15 (14–16, 13–18)	14 (14–15, 11–18)	17 (15–18, 12–23) ¹	20 (19–22, 13–36)
No. (%) of patients with a history of fever	34 (100)	71 (100)	118 (100)	411 (98)
No. of patients with a median temp of > 37.5°C (IQR, range)	37.5 (36.7–38.2, 36–40)	37.4 (36.8–38.2, 36–40.1)	37.6 (37–38.5, 36–40.9) ¹	37.3 (36.8–38.1, 36–40.9)
No. of days to fever clearance (median; range)	0 (0–4)	0 (0–3)	1 (0–2)	0 (0–4)
Geometric mean no. of parasites in the blood/μl (range)	3,096 (160–31,149)	3,633 (160–50,240)	4,886 (48–57,274)	3,768 (32–121,581)
No. of days to parasite clearance (median; range)	2 (1–4)	2 (1–3)	2 (1–4)	2 (1–5)
No. (%) of patients with gametocytemia	23 (68)	56 (79)	104 (88)	352 (84)
Median chloroquine level ^c in ng/ml (range)	54.3 (6.4–115)	50.1 (27.3–138)	56.1 (27.6–161)	77.9 (26.9–248)
Median piperazine level ^c in ng/ml (range)	37.5 (15.3–109)	29.4 (10.7–78.2)	35.3 (7.7–131)	37 (7.3–181)
Median carboxyprimaquine level ^c in ng/ml (range)	261 (29–1,410)	406 (9–1,590)	563 (40–2,740)	793 (59–4,710)
Median primaquine level ^c in ng/ml (range)	3.5 (1.1–43.8)	3.8 (0.9–349)	5.2 (0.9–106)	6.9 (1–293)
Median carboxyprimaquine/primaquine ratio ^c (range)	86 (12–207)	97 (4–256)	98 (23–516)	106 (2–564)

^aIQR, interquartile range. Primaquine-7 dose, 1 mg/kg/day for 7 days; primaquine-14 dose, 0.5 mg/kg/day for 14 days.^bA numeric superscript denotes the number of missing patient values.^cOnly data from the first episode are included.

FIG 1 Effect of age on primaquine and carboxyprimaquine exposures in patients with acute vivax malaria. (A) Age versus mg/kg primaquine daily dose; (B) age versus the primaquine plasma concentration on day 7; (C) age versus the plasma carboxyprimaquine concentration on day 7; (D) age versus the ratio of the day 7 carboxyprimaquine to primaquine concentrations. The black lines show model fits using a generalized additive model with a smooth spline term for age, with adjustment for the daily mg/kg dose, the number of days of primaquine received, the time since the last dose, the partner drug, and the fever clearance time (dashed line, mean predicted concentration for 1 mg/kg daily; solid line, mean predicted concentration for 0.5 mg/kg daily).

confidence intervals [CI], 1.9 to 3.1; $P = 0.0003$) and a 2.0-fold increase in day 7 plasma carboxyprimaquine concentrations (95% CI, 1.7 to 2.4; $P = 0.0002$). A 10-fold increase in the plasma piperaquine day 7 concentrations was associated with a 2.5-fold increase in primaquine concentrations (95% CI, 1.9 to 3.2; $P = 0.0005$) and a 2.5-fold increase in carboxyprimaquine concentrations on day 7 (95% CI, 2.1 to 3.0; $P = 10^{-7}$). Scatterplots showing the univariate relationships between the partner drug concentrations and primaquine/carboxyprimaquine concentrations are shown in Fig. S2 in the supplemental material. Treatment with chloroquine or piperaquine had no statistically significant effect on the carboxyprimaquine/primaquine ratios.

Effect of CYP 2D6 genotypes. *CYP 2D6* genotypes were determined for 154 patients. The estimated *CYP 2D6* allele frequencies in this population were as follows: 25% for allele *1, 23% for *2, 2% for *4, 10% for *5, 38% for *10, 0.5% for *39, and 1% for *36. Alleles *4, *5, and *36 are considered null-metabolizer alleles (enzyme activity score of 0); *10 is a poor-metabolizer allele (activity score of 0.25), and *1 and *39 are normal-metabolizer alleles (activity score of 1). In total, there were 3 patients with an activity score of 0 (nonmetabolizers) and 36 patients with an activity score of either 0.25 or 0.5 (poor metabolizers). For primaquine day 7 plasma concentrations, model residuals (defined as the observed concentration minus the predicted concentration, both on the log scale, where the model is based on age, milligram-per-kilogram dose, fever clearance, time since last dose, number of days of dosing, and partner drug) were correlated significantly with the genotype-defined *CYP 2D6* enzyme activity scores (Fig. 2) ($\rho = -0.2$, $P = 0.004$). Patients with low *CYP 2D6* activity scores had, on average, higher observed concentrations of primaquine than predicted (i.e., positive

FIG 2 Relationship between *CYP 2D6* genotypes and primaquine and carboxyprimaquine exposures, and day 7 methemoglobin levels. (A and B) The y axis shows the model residuals (observed minus predicted) for the main model of exposure (adjusting for age, daily mg/kg primaquine dose, partner drug, and febrile clearance time). (C) The y axis shows the observed day 7 methemoglobin percentage. In all three panels, the x axis is the activity score predicted from the *CYP 2D6* diplotype (26). Each box-and-whisker plot shows the median value, the interquartile range, and the range. The box widths are proportional to the square root of the number of observations per activity score.

residuals), whereas patients with normal activity scores had lower concentrations than expected (i.e., negative residuals). For plasma carboxyprimaquine concentrations, a trend in the same direction was observed, but the correlation was substantially smaller, and it was not significant ($P = 0.1$).

Methemoglobinemia and hemolysis. Primaquine causes predictable methemoglobinemia. As reported previously, methemoglobin concentrations rose over the first week of primaquine treatment and then plateaued in the 14-day primaquine groups. The day 7 values were usually the peak recorded values (Fig. S3). Methemoglobin (expressed as a percentage of the hemoglobin concentration) was lower in children than in young adults ($P < 10^{-16}$ for a spline term fit to age in a generalized additive regression model) (Fig. 3A) and was lower in patients with loss-of-function *CYP 2D6* polymorphisms (Fig. 2C shows an absolute decrease of 1.2 percentage points in the mean day 7 methemoglobin level in patients with an activity score of ≤ 0.5 compared to patients with an activity score of > 0.5 [95% CI, 0.1 to 2.3%; $P = 0.04$]). Day 7 methemoglobin levels were associated with both primaquine and carboxyprimaquine day 7 plasma concentrations. A 10-fold increase in primaquine day 7 concentrations was associated with an absolute increase of 0.7 percentage point in day 7 methemoglobin levels (95% CI, 0.14 to 1.23), and a 10-fold

FIG 3 Age and drug exposure relationships with hemolysis and methemoglobinemia. (A) Age and day 7 methemoglobin; (B) age and the day 7 change in hematocrit from baseline; (C) day 7 methemoglobin and predose primaquine concentrations; (D) day 7 methemoglobin and predose carboxyprimaquine concentrations. (A and B) The black lines show model fits under a generalized additive model with a smooth spline term for age and with adjustment for daily mg/kg dose, the number of days of primaquine received, the time since the last dose, and G6PD status (dashed line, mean predicted concentration for 1 mg/kg daily; solid line, mean predicted concentration for 0.5 mg/kg daily).

increase in carboxyprimaquine concentrations was associated with an absolute increase of 1.1 percentage points in day 7 methemoglobin levels (95% CI, 0.5 to 2.0). Four patients stopped taking primaquine because of elevated methemoglobin levels and associated symptoms. Two of these patients stopped on day 5 and, as the day 6 primaquine dose was not administered, they had low day 7 primaquine and carboxyprimaquine levels. One of these patients had a recorded methemoglobin level of 20.9% on day 6, followed by 18.1% on day 7. The other three patients had peri-oral cyanosis (blue lips). Their peak recorded methemoglobin levels were between 10 and 12%.

There was no relationship between age and hemolysis, defined as the percentage drop in hematocrit between baseline and day 7 (Fig. 3B).

Primaquine exposure and *P. vivax* recurrence. We did an exploratory analysis of the relationship between primaquine exposures and times to the first *P. vivax* recurrence. A total of 564 patients had recorded day 7 drug levels and methemoglobin levels, and 70 had at least one recurrence during follow-up. After adjustment for age and partner drug, day 7 concentrations of primaquine and carboxyprimaquine were not associated with the risk of recurrence, but a 1% absolute increase in day 7 methemoglobin was associated with a hazard ratio for recurrence of 0.9 (95% CI, 0.85 to 0.99; $P = 0.02$). Two of the three patients with a *CYP 2D6* activity score of 0 had a *P. vivax* recurrence, and one of them had 2 recurrences (the interval between recurrences was >65 days). Studies are ongoing to characterize the relationship between *CYP 2D6* genotypes and the risk of *P. vivax* relapse in this population.

DISCUSSION

Administration of therapeutic doses of 8-aminoquinoline antimalarials (radical cure) to patients with *P. vivax* malaria is necessary to prevent relapses. Relapses of vivax malaria are a major cause of morbidity and developmental delay in children living in areas of endemicity, so the optimization of primaquine dosing is critical for this age group. The higher *P. vivax* relapse rates in children than in adults living in areas of endemicity observed in some studies (17) are attributed usually to lower levels of immunity, but lower drug exposures may also be a contributory factor. For many antimalarial drugs, exposures in children are lower than in adults, and an increase in the weight-adjusted dosing is needed (1). The limited pharmacokinetic evidence to date for primaquine does not provide a clear picture, with a study from Papua New Guinea suggesting little difference between adults and older children (2), whereas both a study of single-dose primaquine (used as a *P. falciparum* gametocytocide) in Tanzania (3) and a study of primaquine for a radical *P. vivax* cure in Brazil (4) suggest that younger children had lower exposures. The relationship between plasma primaquine concentrations and antimalarial effect is complex. Primaquine is metabolized rapidly (elimination half-life, ~5 h) via monoamine oxidase to a biologically inert metabolite, carboxyprimaquine, and through a separate pathway, mainly via *CYP 2D6*, to several active hydroxylated metabolites (6, 18). The exact mechanism of antimalarial action remains uncertain. Laboratory studies suggest that these unstable hydroxylated metabolites are oxidized to quinoneimines, generating local hydrogen peroxide, which is parasitocidal. The quinoneimines in turn are substrates for cytochrome P450 NADPH:oxidoreductase, resulting in hydrogen peroxide accumulation and augmenting the parasitocidal effect (19). These reactions may mediate both the antimalarial therapeutic effects and the oxidant hemolytic adverse effects. Alternatively, the quinonimine itself may cause the hemolytic and possibly parasitocidal effects by forming irreversible ligands with heme. There is also evidence for synergy between the 8-aminoquinolines and other quinoline antimalarials in liver-stage and blood-stage activities (20, 21). The 8-aminoquinolines have innate schizonticidal activity as well as radical curative activity, and 4-aminoquinoline antimalarials increase primaquine concentrations (15, 16, 21). Unfortunately, although the metabolic pathway to the production of the active primaquine metabolites has been characterized *ex vivo*, no validated measure of their concentrations or of their more stable metabolites in whole blood, plasma, or urine has yet been correlated with antimalarial therapeutic responses *in vivo*. Thus, measurement of primaquine and carboxyprimaquine concentrations does not provide a direct correlate of antimalarial activity. Furthermore, because the apparent volume of distribution of carboxyprimaquine is not known, the proportion of parent drug that passes through this inactive metabolic pathway cannot be estimated.

This large study of day 7 trough primaquine and carboxyprimaquine plasma concentrations shows clearly that exposures to both the parent compound and inactive metabolite are lower in children than in adults. This is unlikely to be explained by lower oral bioavailability, as primaquine is generally well absorbed, the children in the study were clinically well by 7 days after starting treatment (14), all doses were observed, and only 7 children who were ≤ 10 years old (≤ 27 kg) vomited. The lower concentrations in children presumably reflects either larger primaquine (and carboxyprimaquine) distribution volumes or increased clearance or both. The lower ratio of carboxyprimaquine to primaquine in children may also be explained by these age-related pharmacokinetic changes. Although greater diversion down the bioactivating *CYP* pathway cannot be excluded as an explanation for these findings, there is no convincing evidence for augmented drug efficacy (e.g., no relationship with fever or parasite clearance times) or increased hemolytic toxicity in children to support this. Furthermore, in this study, methemoglobinemia was lower in children than in young adults, indicating that the formation of oxidant metabolites was reduced (22). Lower methemoglobin concentrations were associated with an increased risk of *P. vivax* recurrence. The importance of *CYP 2D6* primaquine bioactivation in the generation of oxidant metabolites was evidenced by the lower levels of methemoglobinemia in patients with loss-of-function *CYP 2D6* polymorphisms (Fig. 2C).

Taken together, this evidence strongly suggests that children have lower exposures to the biologically active metabolites of primaquine than do adults receiving the same weight-adjusted doses.

This study was sufficiently large that the influence of other covariates affecting primaquine exposures could be examined. We have reported previously that coadministration of quinoline or structurally related antimalarials increases primaquine and carboxyprimaquine concentrations (15, 16). This was confirmed in this study. The effects on the parent compound of chloroquine and piperazine were similar, whereas piperazine had a larger effect on carboxyprimaquine. In Asia, loss-of-function *CYP 2D6* genetic polymorphisms (mainly the *10 allele) are common. In this study, low *CYP 2D6* activity scores (derived from the genotype) were associated with higher primaquine levels and lower levels of methemoglobinemia, as was expected from reduced metabolic conversion through this bioactivation pathway.

There are several limitations to this study. This analysis is limited to drug concentrations at one time point during radical-cure treatment, providing a limited description of drug exposures. Very young children are underrepresented, and the analysis does not include any infants of <1 year old. In addition, *CYP 2D6* genotyping was not performed for all patients.

There is no evidence that the adverse effects of primaquine are significantly worse in children than in adults. The main therapeutic implication of this large pharmacometric evaluation is that children may require larger weight-adjusted doses of primaquine than adults.

MATERIALS AND METHODS

Clinical trial. This study was conducted by the Shoklo Malaria Research Unit (SMRU), which is located on the northwest Thailand-Myanmar border. Full details of the study have been reported previously (14), and the trial has been registered at ClinicalTrials.gov (NCT01640574). In brief, patients who presented to an outpatient clinic with microscopy-confirmed uncomplicated *P. vivax* mono-infections were enrolled if they were ≥ 6 months old and if they were glucose-6-phosphate dehydrogenase (G6PD) normal by the fluorescent spot test. Patients were excluded if they were pregnant, breastfeeding an infant who was ≤ 6 months of age, had a hematocrit of $\leq 25\%$, or had received a blood transfusion within 3 months. Written informed consent was obtained from patients or from parents or caregivers of children below 18 years old.

Patients were randomized 1:1:1:1 into each of the following groups:

1. Chloroquine (10 mg base/kg on the first two days of treatment and then 5 mg base/kg on day 3 [Remedica, Ltd., Cyprus]) and primaquine (1 mg base/kg/day for 7 days [Government Pharmaceutical Organization, Thailand]).
2. Chloroquine as described above and primaquine (0.5 mg base/kg/day for 14 days).
3. Dihydroartemisinin-piperazine (dihydroartemisinin at 7 mg/kg and piperazine at 56 mg/kg for 3 days [Guilin Pharmaceutical Company, China]) and primaquine (1 mg base/kg/day for 7 days).
4. Dihydroartemisinin-piperazine as described above plus primaquine (0.5 mg base/kg/day for 14 days).

Food and drink were given before drug administration. Primaquine was usually started on the same day as the schizonticide (i.e., the first day of treatment), but in a subset of patients, it was started on the third day of treatment. Patients were assessed daily while on treatment. Methemoglobin was measured either daily or on days 1, 3, 6, and 13 and additionally on day 10 in the primaquine 14-day groups by means of a transcutaneous pulse oximeter (Masimo Radical-7). One recruitment clinic did not have access to a transcutaneous oximeter, so 80 study patients did not have methemoglobin measurements. All drug doses were supervised.

A venous blood sample for antimalarial drug levels was taken before drug administration on day 6 (i.e., the 7th day of treatment) ± 2 days, and plasma was separated and stored at -70°C . Patients were followed at weeks 2 and 4 and then every 4 weeks until 52 weeks. If there was a *P. vivax* recurrence then a venous plasma blood sample for drug levels was taken again. Standard high-dose primaquine (0.5 mg base/kg/day for 14 days) and chloroquine were given for the treatment of recurrences. Follow-up was restarted as if the patient had been newly recruited, and day 6 sampling for drug levels was collected again. The total study duration was 52 weeks from enrollment.

Drug measurements. Chloroquine/desethylchloroquine, piperazine, and primaquine/carboxyprimaquine plasma concentrations were measured using three validated liquid chromatography (LC)-tandem mass spectrometry (MS/MS) methods (15, 16, 23). The lower limits of quantification (LLOQ) were set to 1.4, 1.5, 1.14, and 4.88 ng/ml for chloroquine/desethylchloroquine, piperazine, primaquine, and carboxyprimaquine, respectively. All three quantification methods were validated according to regulatory standards, and three levels of quality control samples were analyzed in triplicate within each batch of clinical samples. Total imprecision (i.e., relative standard deviation) for all quality control samples was below 10% during drug quantification.

CYP 2D6 genotyping. We carried out a nested case-control genotyping study to assess the effect of *CYP 2D6* polymorphisms on recurrent vivax malaria. Cases were all patients with a recorded recurrent episode of vivax malaria during follow-up, and controls were patients without observed recurrences matched by age and sex (in total, 158 patients). The *CYP 2D6* gene duplications/multiplications and gene deletions were determined by extra-long-range PCR (XL-PCR) using a previously published protocol (24). The functional *CYP 2D6* and nonfunctional *CYP2D8* and *CYP2D7* genes were then differentiated by intron 2 (INT2) sequencing. The variants *1, *2, *4, *5, *10, *39, and *36 were identified by rapid identification of four polymorphic loci with allele-specific oligonucleotide probes using real-time single nucleotide polymorphism (SNP) genotyping. From the *CYP 2D6* genotyping result, the *CYP 2D6* activity score was obtained as described previously (15, 25–27).

Ethics approval. This study was approved by both the Mahidol University Faculty of Tropical Medicine Ethics Committee (MUTM 2011-043, TMEC 11-008) and the Oxford Tropical Research Ethics Committee (OXTREC 17-11).

Statistical analysis. All drug concentration data were analyzed on the log base 10 scale. Three main generalized additive linear regression models were fitted to the $\log_{10}$ primaquine day 7 concentrations, the $\log_{10}$ carboxyprimaquine concentrations, and the $\log_{10}$ carboxyprimaquine-to-primaquine ratios. We used the following predictors: age (years), milligram-per-kilogram daily dose of primaquine (the administered milligram-per-kilogram dose, not the target dose), study arm (DHA-piperaquine versus chloroquine), fever clearance time (days), the number of days since the start of primaquine dosing (6 versus 4 days), and the time since the previous primaquine dose (in hours). We excluded drug measurements taken outside an 18- to 30-h window after the previous dose (only trough levels approximately 1 day after dosing), except for those for which the previous dose was given less than 30 min before the drug measurement. More than 90% of samples were taken in this window (see Fig. S1 in the supplemental material). A smooth spline term with 3 degrees of freedom was used to estimate nonlinear effects of age. Individual random intercept terms were specified to account for multiple day 7 measurements in the same patient. To assess any residual effect of *CYP 2D6* diplotypes, we fitted linear regression models to the model residual concentrations with the *CYP 2D6* activity score as a linear predictor. To estimate the effect of the partner drug concentrations (not available for every day 7 measurement), we fitted separate generalized additive regression models to the primaquine and carboxyprimaquine $\log_{10}$ concentrations with either the $\log_{10}$ chloroquine plus desethylchloroquine (assuming equal antimalarial activities of the parent drug and metabolite) concentration or the $\log_{10}$ piperaquine concentration as additional predictors.

We fitted a generalized additive regression model to the day 7 blood methemoglobin concentrations (expressed as a percentage of the hemoglobin concentration) with age, milligram-per-kilogram daily dose of primaquine, study arm (DHA-piperaquine versus chloroquine), fever clearance time (days), and days since the start of primaquine dosing as predictors. A spline term with 3 degrees of freedom was fit to age. Secondary models included the *CYP 2D6* activity score (available only for the subset of patients in the nested case-control investigation) as an additional covariate, both on the linear scale and as a binary covariate (≤ 0.5 versus > 0.5). The activity score has an arbitrary scale, so it is unclear how best to encode it in regression models (i.e., an activity score of 2 versus 1 is not the same as 1 versus 0).

We fitted Cox proportional hazard models to the time to the first recurrence (right censored event for patients who did not have a recurrence during follow-up), with the following continuous predictors: age, partner schizonticidal drug (chloroquine versus piperaquine), and either the $\log_{10}$ primaquine or the $\log_{10}$ carboxyprimaquine day 7 concentration (they are highly colinear, so we fitted two separate models). Additional models included the day 7 methemoglobin levels as an additional predictor (measured at all sites except the one where a transcutaneous pulse oximeter was not available).

The generalized additive models were fitted in R version 4.0.2 using the package *mgcv*, version 1.8. The Cox proportional hazard model used the *survival* package, version 3.2. Code in the form of an RMarkdown script along with all analyzed data are available in an accompanying GitHub repository for full reproducibility (https://github.com/jwatowatson/Primaquine_day7).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

ACKNOWLEDGMENTS

We thank the SMRU staff for their hard work in running this study and the MORU Clinical Pharmacology Laboratory in Bangkok, Thailand, for their diligent work in sample processing and drug measurements. Special appreciation goes to the contributions of Htet Htet Chaw, Nwe Wah Lin, Thida Zin, Say Paw, Naw Eh Shee, Thu Lay Paw, Rattanaorn Raksapraidee, Moo Koh Paw, the data entry team headed by Jacher Wiladphaingern, the logistics team, and the laboratory teams headed by Kanlaya Sriprawat and Germana Bancone.

We also thank the Data Safety and Monitoring Board, namely, Bob Taylor, Charles Woodrow, and Nicholas Day, for their time and commitment.

This work was supported by the Wellcome Trust (grant 089179/Z/09/Z to N.J.W.). A CC BY or equivalent license is applied to the author accepted manuscript arising from this submission, in accordance with the grant's open access conditions. C.S.C., J.A.W., A.P.P., J.T., C.L., F.H.N., and N.J.W. are supported by the Wellcome Trust (program grant 089179). C.S.C., J.A.W., A.P.P., J.T.,

C.L., F.H.N., and N.J.W. are part of the Wellcome Trust Mahidol University Oxford Tropical Medicine Research Program funded by the Wellcome Trust.

REFERENCES

- World Health Organization. 2015. Guidelines for the treatment of malaria, 3rd ed. World Health Organization, Geneva, Switzerland.
- Moore BR, Salman S, Benjamin J, Page-Sharp M, Robinson LJ, Waita E, Batty KT, Siba P, Mueller I, Davis TME, Betuela I. 2014. Pharmacokinetic properties of single-dose primaquine in Papua New Guinean children: feasibility of abbreviated high-dose regimens for radical cure of vivax malaria. *Antimicrob Agents Chemother* 58:432–439. <https://doi.org/10.1128/AAC.01437-13>.
- Gonçalves BP, Pett H, Tiono AB, Murry D, Sirima SB, Niemi M, Bousema T, Drakeley C, Ter Heine R. 2017. Age, weight, and CYP2D6 genotype are major determinants of primaquine pharmacokinetics in African children. *Antimicrob Agents Chemother* 61:e02590-16. <https://doi.org/10.1128/AAC.02590-16>.
- Vieira MVDF, Matos Lopes TR, Mello AGNC, de Sena LWP, Commons RJ, Vieira JLF. 2020. Doses of primaquine administered to children with Plasmodium vivax according to an age-based dose regimen. *Pathog Glob Health* 114:388–392. <https://doi.org/10.1080/20477724.2020.1799166>.
- Gogtay N, Karande S, Kadam P, Momin S, Thatte U. 2021. Evaluation of pharmacokinetics of single-dose primaquine in undernourished versus normally nourished children diagnosed with Plasmodium vivax malaria in Mumbai. *J Postgrad Med* 67:75–79. https://doi.org/10.4103/jpgm.JPGM_1254_20.
- Pybus BS, Marcsisin SR, Jin X, Deye G, Sousa JC, Li Q, Caridha D, Zeng Q, Reichard GA, Ockenhouse C, Bennett J, Walker LA, Ohrt C, Melendez V. 2013. The metabolism of primaquine to its active metabolite is dependent on CYP 2D6. *Malar J* 12:e212. <https://doi.org/10.1186/1475-2875-12-212>.
- Neafsey P, Ginsberg G, Hattis D, Sonawane B. 2009. Genetic polymorphism in cytochrome P450 2D6 (CYP2D6): population distribution of CYP2D6 activity. *J Toxicol Environ Health B Crit Rev* 12:334–361. <https://doi.org/10.1080/10937400903158342>.
- McGraw J, Waller D. 2012. Cytochrome P450 variations in different ethnic populations. *Expert Opin Drug Metab Toxicol* 8:371–382. <https://doi.org/10.1517/17425255.2012.657626>.
- Puaprasert K, Chu C, Saralamba N, Day NPJ, Nosten F, White NJ, Dondorp AM, Imwong M. 2018. Real time PCR detection of common CYP2D6 genetic variants and its application in a Karen population study. *Malar J* 17:427. <https://doi.org/10.1186/s12936-018-2579-8>.
- Bennett JW, Pybus BS, Yadava A, Tosh D, Sousa JC, McCarthy WF, Deye G, Melendez V, Ockenhouse CF. 2013. Primaquine failure and cytochrome P-450 2D6 in Plasmodium vivax malaria. *N Engl J Med* 369:1381–1381. <https://doi.org/10.1056/NEJMc1301936>.
- Baird JK, Louisa M, Noviyanti R, Ekawati L, Elyazar I, Subekti D, Chand K, Gayatri A, Soebianto S, Crenna-Darusallam C, Djoko D, Hasto BD, Meriyenes D, Wesche D, Nelwan EJ, Sutanto I, Sudoyo H, Setiabudy R, Instiaty. 2018. Association of impaired cytochrome P450 2D6 activity genotype and phenotype with therapeutic efficacy of primaquine treatment for latent Plasmodium vivax malaria. *JAMA Netw Open* 1:e181449. <https://doi.org/10.1001/jamanetworkopen.2018.1449>.
- Price RN, von Seidlein L, Valecha N, Nosten FH, Baird JK, White NJ. 2014. Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. *Lancet Infect Dis* 14:982–991. [https://doi.org/10.1016/S1473-3099\(14\)70855-2](https://doi.org/10.1016/S1473-3099(14)70855-2).
- Taylor WRJ, Thriemer K, von Seidlein L, Yuentrakul P, Assawariyathipat T, Assefa A, Auburn S, Chand K, Chau NH, Cheah PY, Dong LT, Dhorda M, Degaga TS, Devine A, Ekawati LL, Fahmi F, Hailu A, Hasanzai MA, Hien TT, Khu H, Ley B, Lubell Y, Marfurt J, Mohammad H, Moore KA, Naddim MN, Pasaribu AP, Pasaribu S, Promnarate C, Rahim AG, Sirithiranont P, Solomon H, Sudoyo H, Sutanto I, Thanh NV, Tuyet-Trinh NT, Waithira N, Woyessa A, Yamin FY, Dondorp A, Simpson JA, Baird JK, White NJ, Day NP, Price RN. 2019. Short-course primaquine for the radical cure of Plasmodium vivax malaria: a multicentre, randomised, placebo-controlled non-inferiority trial. *Lancet* 394:929–938. [https://doi.org/10.1016/S0140-6736\(19\)31285-1](https://doi.org/10.1016/S0140-6736(19)31285-1).
- Chu CS, Phyo AP, Turner C, Win HH, Poe NP, Yotyingaphiram W, Thinnaraw S, Wilairisak P, Raksapraddee R, Carrara VI, Paw MK, Wiladphaingern J, Proux S, Bancone G, Sriprawat K, Lee SJ, Jeeyapant A, Watson JA, Tarning J, Imwong M, Nosten F, White NJ. 2019. Chloroquine versus dihydroartemisinin-piperaquine with standard high-dose primaquine given either for 7 days or 14 days in Plasmodium vivax malaria. *Clin Infect Dis* 68:1311–1319. <https://doi.org/10.1093/cid/ciy735>.
- Hanboonkunupakarn B, Ashley EA, Jittamala P, Tarning J, Pukrittayakamee S, Hanpithakpong W, Chotsiri P, Wattanakul T, Panapipat S, Lee SJ, Day NPJ, White NJ. 2014. Open-label crossover study of primaquine and dihydroartemisinin-piperaquine pharmacokinetics in healthy adult Thai subjects. *Antimicrob Agents Chemother* 58:7340–7346. <https://doi.org/10.1128/AAC.03704-14>.
- Pukrittayakamee S, Tarning J, Jittamala P, Charunwatthana P, Lawpoolsri S, Lee SJ, Hanpithakpong W, Hanboonkunupakarn B, Day NPJ, Ashley EA, White NJ. 2014. Pharmacokinetic interactions between primaquine and chloroquine. *Antimicrob Agents Chemother* 58:3354–3359. <https://doi.org/10.1128/AAC.02794-13>.
- Luxemburger C, van Vugt M, Jonathan S, McGready RM, Looareesuwan S, White NJ, Nosten FH. 1999. Treatment of vivax malaria on the western border of Thailand. *Trans R Soc Trop Med Hyg* 93:433–438. [https://doi.org/10.1016/S0035-9203\(99\)90149-9](https://doi.org/10.1016/S0035-9203(99)90149-9).
- Avula B, Tekwani BL, Chaurasiya ND, Fasinu P, Dhammika Nanayakkara NP, Bhandara Herath HMT, Wang Y-H, Bae J-Y, Khan SI, Elshohly MA, McChesney JD, Zimmerman PA, Khan IA, Walker LA. 2018. Metabolism of primaquine in normal human volunteers: investigation of phase I and phase II metabolites from plasma and urine using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *Malar J* 17:294. <https://doi.org/10.1186/s12936-018-2433-z>.
- Camarda G, Jirawatcharadech P, Priestley RS, Saif A, March S, Wong MHL, Leung S, Miller AB, Baker DA, Alano P, Paine MJ, Bhatia SN, O'Neill PM, Ward SA, Biagini GA. 2019. Antimalarial activity of primaquine operates via a two-step biochemical relay. *Nat Commun* 10:3226–3229. <https://doi.org/10.1038/s41467-019-11239-0>.
- Alving AS, Arnold J, Hockwald RS, Clayman CB, Dern RJ, Beutler E, Flanagan CL. 1955. Potentiation of the curative action of primaquine in vivax malaria by quinine and chloroquine. *J Lab Clin Med* 46:301–306.
- Baird JK. 2019. 8-Aminoquinoline therapy for latent malaria. *Clin Microbiol Rev* 32:1–68. <https://doi.org/10.1128/CMR.00011-19>.
- Umbreit J. 2007. Methemoglobin—it's not just blue: a concise review. *Am J Hematol* 82:134–144. <https://doi.org/10.1002/ajh.20738>.
- Kaewkhao K, Chotivanich K, Winterberg M, Day NP, Tarning J, Blessborn D. 2019. High sensitivity methods to quantify chloroquine and its metabolite in human blood samples using LC-MS/MS. *Bioanalysis* 11:333–347. <https://doi.org/10.4155/bio-2018-0202>.
- Dorado P, Cáceres MC, Pozo-Guisado E, Wong ML, Licinio J, Llerena A. 2005. Development of a PCR-based strategy for CYP2D6 genotyping including gene multiplication of worldwide potential use. *Biotechniques* 39:571–574.
- Gaedigk A, Simon SD, Pearce RE, Bradford LD, Kennedy MJ, Leeder JS. 2008. The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. *Clin Pharmacol Ther* 83:234–242. <https://doi.org/10.1038/sj.clpt.6100406>.
- Caudle KE, Sangkuhl K, Whirl-Carrillo M, Swen JJ, Haidar CE, Klein TE, Gammal RS, Relling MV, Scott SA, Hertz DL, Guchelaar HJ, Gaedigk A. 2020. Standardizing CYP2D6 genotype to phenotype translation: consensus recommendations from the Clinical Pharmacogenetics Implementation Consortium and Dutch Pharmacogenetics Working Group. *Clin Transl Sci* 13:116–124. <https://doi.org/10.1111/cts.12692>.
- Spring MD, Lon C, Sok S, Sea D, Wojnarski M, Chann S, Kuntawunginn W, Heng TK, Nou S, Arsanok M, Sriwichai S, Vanachayangkul P, Lin JT, Manning JE, Jongsakul K, Pichyangkul S, Satharath P, Smith PL, Dysoley L, Saunders DL, Waters NC. 2020. Prevalence of CYP2D6 genotypes and predicted phenotypes in a cohort of Cambodians at high risk for infections with Plasmodium vivax. *Am J Trop Med Hyg* 103:756–759. <https://doi.org/10.4269/ajtmh.20-0061>.