MAJOR ARTICLE

Residual Antimalarial Concentrations before Treatment in Patients with Malaria from Cambodia: Indication of Drug Pressure

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Background. The Thai-Cambodian border has been known as the origin of antimalarial drug resistance for the past 30 years. There is a highly diverse market for antimalarials in this area, and improved knowledge of drug pressure would be useful to target interventions aimed at reducing inappropriate drug use.

Methods. Baseline samples from 125 patients with falciparum malaria recruited for 2 in vivo studies (in Preah Vihear and Pursat provinces) were analyzed for the presence of 14 antimalarials in a single run, by means of a liquid chromatography–tandem mass spectrometry assay.

Results. Half of the patients had residual drug concentrations above the lower limit of calibration for at least 1 antimalarial at admission. Among the drugs detected were the currently used first-line drugs mefloquine (25% and 35% of patients) and piperaquine (15% of patients); the first-line drug against vivax malaria, chloroquine (25% and 41% of patients); and the former first-line drug, quinine (5% and 34% patients).

Conclusions. The findings demonstrate that there is high drug pressure and that many people still seek treatment in the private and informal sector, where appropriate treatment is not guaranteed. Promotion of comprehensive behavioral change, communication, community-based mobilization, and advocacy are vital to contain the emergence and spread of parasite resistance against new antimalarials.

A few years ago, the World Health Organization (WHO) recommended that conventional monotherapies—such as chloroquine, amodiaquine, and sulfadoxine-pyrimethamine—for the treatment of falciparum malaria should be replaced by artemisinin-based combination therapies (ACTs), and today ACTs are used as first-line treatment throughout the world [1–4]. Initiatives to scale up control interventions and eliminate malaria are critically dependent on the sustained efficacy of ACTs. However, there is recent worrying evidence of a reduced response to artemisinins arising on the Thai-Cambodian border [1, 5–13]. This area has long been known as the origin of antimalarial drug resistance. Parasites carrying resistance markers against chloroquine, sulfadoxine-pyrimethamine, and mefloquine were first found here before they appeared elsewhere in the world [5, 6, 14]. In Cambodia, because of poor transportation and public health infrastructure, antimalarials were made available in the private sector to increase access to the drug. While instrumental to reaching out to more patients, this also led to uncontrolled use of these drugs. A recent study of malaria treatment–seeking behavior in Cambodia showed that >80% of patients initially sought treatment from private providers and pharmacies or consumed drugs on their own [15]. Drugs from the private sector are often of substandard quality, and the drug providers do not emphasize adherence. These key factors lead to treatment failure and the development of resistance against chloroquine, sulfadoxine-pyrimethamine, and mefloquine in this area, and it is conceivable that the availability

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of artemisinin monotherapies, incorrect dosages, and poor drug quality affected the response to artemisinin derivatives, too [7].

Several studies and programs are currently focusing on improving the availability of quality-assured ACTs. In a study of access to ACTs in remote areas of Cambodia, it was shown that in the private sector up to 26% of patients received chloroquine, 22% received artemisinin monotherapy, and 12% received quinine [16]. Treatment from private practitioners contained artemisinin monotherapy in 34% of cases, chloroquine in 11% of cases, and quinine in 13% of cases. Similar findings were reported for public health facilities, where artesunate monotherapy and chloroquine each accounted for 11% of the treatments given. Provision of free diagnosis and treatment through trained village malaria workers were found to be effective means of increasing ACT coverage in the studied settings. In 2008, a baseline outlet and household survey was conducted in Cambodia within the framework of ACTwatch [17]. The survey assessed levels and trends in the availability, price, and volume of antimalarials; providers' perceptions and knowledge of antimalarials; consumer treatment-seeking behavior; and volumes of specific antimalarials consumed. The findings are expected to provide and promote evidence and recommendations for policy makers on methods to increase availability and decrease the consumer price of quality-assured ACT through the private sector. WHO and several key partners from the ministries of health and academia currently work on containing the spread of artemisinin-resistant malarial parasites along the Thai-Cambodian border. One of the key objectives of the collaboration is to support the containment and elimination of artemisinin-tolerant parasites through comprehensive behavioral change, communication, community-based mobilization, and advocacy [8, 9]. Knowledge of drug use in this specific area could help in evaluating the effect of the interventions undertaken. The present study aims at gathering information about circulating antimalarials in 2 Cambodian provinces with different levels of drug resistance and, thus, different recommended first-line treatments. It was part of a multinational project aimed at assessing the effect of the pharmacogenetic profile on the pharmacokinetics of standard antimalarials (to be reported elsewhere). Here, we report findings from the analysis of baseline samples from patients with malaria recruited from 2 in vivo studies for the presence of 14 currently-in-use antimalarials in a single run, by means of a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay [18].

METHODS

Study areas and populations. The study was performed in 2 rural areas with moderate transmission intensity during the rainy season. Artesunate-mefloquine (Malarine) has been the first-line treatment for *Plasmodium falciparum* infection since 2000 and was available at government health facilities as well as in private structures through a social marketing program. Chloroquine has been used as first-line treatment for *Plasmodium vivax* infection. Artesunate, dihydroartemisinin, and quinine can easily be found in the private sector without a doctor's prescription. At the time of the second study, dihydroartemisinin-piperaquine had been recently introduced as first-line treatment in the study province because of low clinical efficacy of the artesunate-mefloquine regimen and was available only at government health facilities to ensure controlled use.

Clinical procedures. The trial was based on the standard WHO protocol for in vivo testing [19]. The first study was performed from October 2007 through February 2008 at the Phnom Dék health center (Rovieng district, Preah Vihear province, Cambodia), and the second study was performed from July through October 2008 at the Pramoy health center (Veal Veng district, Pursat province, Cambodia). Respectively, 234 and 287 suspected malaria cases were screened by microscopy. Of these, 67 (29%) and 82 (29%) were infections with *P. falciparum*, and 74 (32%) and 50 (17%) were infections with *P. vivax*. No mixed infections were detected. Adequate parasitemia for enrollment in low to moderate transmission areas requires at least 1 parasite for every 6 white blood cells, corresponding to ~1000 asexual parasites/μL. Patients with a positive slide result for *P. falciparum* were then seen by the clinician, who invited them to participate in the study if they were older than 6 months (in Phnom Dék) or 6 years (in Pramoy), not pregnant or lactating, and did not present with danger signs of complicated malaria or any other severe concomitant illness. Prior reported treatment with antimalarial drugs was not considered to be an exclusion criterion. After informed consent was provided by the patient, the baseline sample (day 0; 4.5 mL of venous blood collected in an ethylenediaminetetraacetic acid Vacutainer [Becton Dickinson]) was obtained for thick and thin smears, hematocrit and drug blood concentration measurements, and spotting on filter paper. In Phnom Dék, the national standard first-line treatment introduced in 2000—artesunate (Arsumax; Sanofi-Aventis) and mefloquine (Eloquine; Medochemie) [7]—was given according to age. In Pramoy, patients were treated with dihydroartemisinin-piperaquine (Duo-Cotecxin; Zhejiang Holley Nanhu Pharmaceutical Company) according to age.

Laboratory procedures. Blood samples were immediately aliquoted into whole blood, plasma, and pellets and then stored in liquid nitrogen. The samples were transferred to a −80°C freezer within 1 week. Plasma concentrations of 14 antimalarial drugs and their metabolites—artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, lumefantrine, desbutyl-lumefantrine, piperaquine, pyronaridine, mefloquine, chloroquine, quinine, pyrimethamine, and sulfadoxine—were determined by LC-MS/MS [18]. The lower limits of the calibration range (LLC) in our method were selected as the lowest levels of the calibration

Table 1. Residual Plasma Concentrations of Antimalarials Found before Treatment in 64 Patients with Malaria from Preah Vihear

Antimalarial	No. of patients	ng/mL			
		Mean	Median	Minimum	Maximum
Mefloquine	16	260.3	152.5	12.6	987.0
Chloroquine	16	75.8	30.1	4.1	579.7
Quinine	3	72.1	4312.7	3.5	7550.8

NOTE. No artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, desbutyl-lumefantrine, lumefantrine, piperaquine, pyronaridine, sulfadoxine, or pyrimethamine was found.

curves, which confidently provide a bias and coefficient of variation within ±20%, in accordance with Food and Drug Administration recommendations [20]. All samples were analyzed twice. First, quantitative measurement was performed using calibration and quality control samples; then, for confirmation, qualitative assessment was repeated using a new chromatographic column that had not been exposed to any antimalarial drugs. To exclude contamination and false-positive results, a large set of blank controls was analyzed before the clinical samples on the new column, checking for the absence of specific MS/MS signals for the antimalarials investigated.

Sample size calculation. This sample size was based on the assumptions made for the main multicentric study.

Data management and analysis. Summary statistics and graphs of the residual plasma concentrations of antimalarials found before treatment in the study population were produced using Stata software (version 10.1, intercooled; StataCorp).

Estimation of dose intake time for mefloquine and piperaquine. To estimate the probable timing of drug intake, we compared the plasma concentrations of mefloquine in patients from Preah Vihear and the plasma concentrations of piperaquine in patients from Pursat at baseline (C_0) and on day 14 (C_{14}) after complete treatment with the respective drug among the same patients. We included only patients for whom we had both samples and who complied with the 3-day treatment schedule. Assuming a mean terminal elimination half-life ($t_{1/2}$) of 15 (90% confidence interval [CI], 12.93–17.07) days for mefloquine [21, 22] and of 27.8 (total range, 10.2–216) days for piperaquine [23] and a similar dosage for prestudy exposure and exposure during the study, a back-calculation was done to estimate the intake time before baseline sampling, as follows:

$$\text{intake time} = \ln(C_{14}/C_0) \cdot t_{1/2}/\ln(2) + 14 \text{ days}.$$

Similar calculations were not attempted for the other antimalarials (ie, chloroquine and quinine), because we did not have the respective C_0 and C_{14} values after standardized treatment in the study patients.

Ethical approval. All applied protocols were approved by the ethics committee of the 2 cantons of Basel (Ethikkommission beider Basel) and the responsible local authorities (National Ethics Committee for Health Research, Cambodia). Blood samples were obtained after receiving written informed consent in Khmer from the participants or their responsible guardians.

RESULTS

At the Phnom Dék health center, 64 patients were eligible and willing to participate in the in vivo study. Of these 64 patients, 38 (59.4%) were male and 26 (40.6%) were female; their age ranged from 2 to 57 years (median, 18 years). Parasite densities ranged from 1200 to 160,000 asexual parasites/μL (median, 20,326 asexual parasites/μL). The presence of an antimalarial drug was detected in the plasma of 33 patients (51.6%): 16 (25.0%) had a mefloquine concentration above the LLC of 2.5 ng/mL, 16 (25.0%) had a chloroquine concentration above the LLC of 2.5 ng/mL, and 3 (4.7%) had a quinine concentration above the LLC of 2.5 ng/mL; no other antimalarials tested were detected. All patients reported either not having taken any antimalarials or not knowing. Parasite densities and age distribution were comparable between patients with residual antimalarial concentrations on day 0 and those with no antimalarials—that is, 1200–142,857 asexual parasites/μL (median, 19,600 asexual parasites/μL) and 2–57 years (median, 19 years) versus 3600–160,000 asexual parasites/μL (median, 21,052 asexual parasites/μL) and 3–55 years (median, 16 years). Summary statistics are shown in Table 1, and box plots of residual plasma concentrations are shown in Figure 1. Of the 33 patients with residual drug concentrations, 3 (9.1%) had them for >1 drug: 2 patients had residual chloroquine and mefloquine concen-

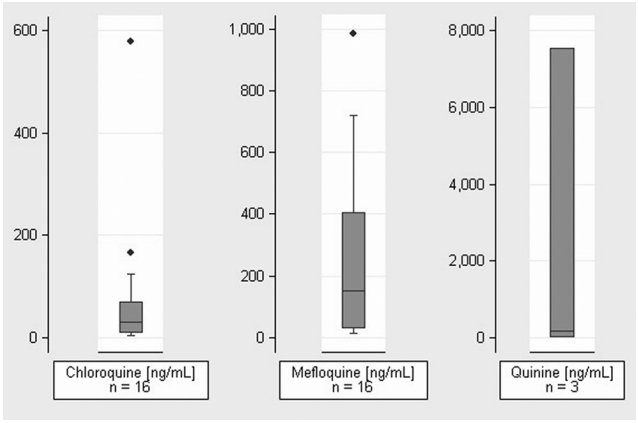

Figure 1. Residual plasma concentrations of antimalarials found before treatment in 64 patients with malaria from Preah Vihear. The number of patients, median, 25th and 75th percentiles, lower and upper adjacent values, and outlier values are shown for chloroquine, mefloquine, and quinine.

trations, and 1 had residual chloroquine and quinine concentrations (Figure 2). Of the 16 patients who had a residual mefloquine concentration, 12 also had a value for day 14; these 12 patients had a median plasma concentration of 52.8 ng/mL (range, 12.6–987 ng/mL) on day 0. One patient had a plasma concentration above the approximate in vivo minimum inhibitory concentration of mefloquine for resistant *P. falciparum* (500 ng/mL) [24, 25]. On day 14, the median plasma concentration was 757 ng/mL (range, 392–1135 ng/mL). This means that a similar dosage level should have been administered at a median of 68 days (interquartile range, 47–79 days; range 17–102 days) before enrollment into the study, to account for the levels observed on day 0. For 2 patients (17%), this estimate was <28 days. Because there were only 3 late parasitological failures on day 42, correlation between day 0 concentration and treatment failure was not assessed. The variability in $t_{1/2}$ translates into 90% confidence intervals extending from 89% to 111% of estimates (median).

At the Pramoy health center, 61 patients were eligible and willing to participate in the in vivo study. Of these 61 patients, 38 (62.3%) were male, and 23 (37.7%) were female; their age ranged from 7 to 53 years (median, 18 years). Parasite densities ranged from 1038 to 219,333 asexual parasites/μL (median, 16,975 asexual parasites/μL). The presence of an antimalarial drug was detected in the plasma of 42 patients (68.9%): 25 (41.0%) had a chloroquine concentration above the LCC of 2.5 ng/mL, 21 (34.4%) had a mefloquine concentration above the LCC of 2.5 ng/mL, 9 (14.7%) had a piperaquine concentration above the LLC of 2 ng/mL, and 2 (3.3%) had a quinine concentration above the LLC of 2.5 ng/mL; no other antimalarials tested were detected. Only 6 patients stated that they had taken antimalarials; the other patients reported not having taken any antimalarials or not knowing. Age distribution was comparable between patients with residual antimalarials on day 0 and those with no antimalarials—that is, 9–53 years (median, 18 years) versus 7–50 years (median, 18 years). As a result of 2 rather extreme values in the group with previous antimalarial treatment, parasite densities differed slightly between the 2 groups—that is, 1038–219,333 asexual parasites/μL (median, 21,395 asexual parasites/μL) in the pretreated group versus 2451–114,666 asexual parasites/μL (median, 9522 asexual parasites/μL) in the group with no antimalarials at inclusion. Summary statistics are shown in Table 2, and box plots of residual plasma concentrations are shown in Figure 3. Of the 42 patients with residual drug concentrations, 12 (28.6%) had them for >1 drug: 1 (8.3%) patient had residual mefloquine and piperaquine concentrations, 1 (8.3%) had residual chloroquine and piperaquine concentrations, 2 (16.7%) had residual mefloquine and quinine concentrations, 5 (41.7%) had residual chloroquine and mefloquine concentrations, and 3 (25.0%) had re-

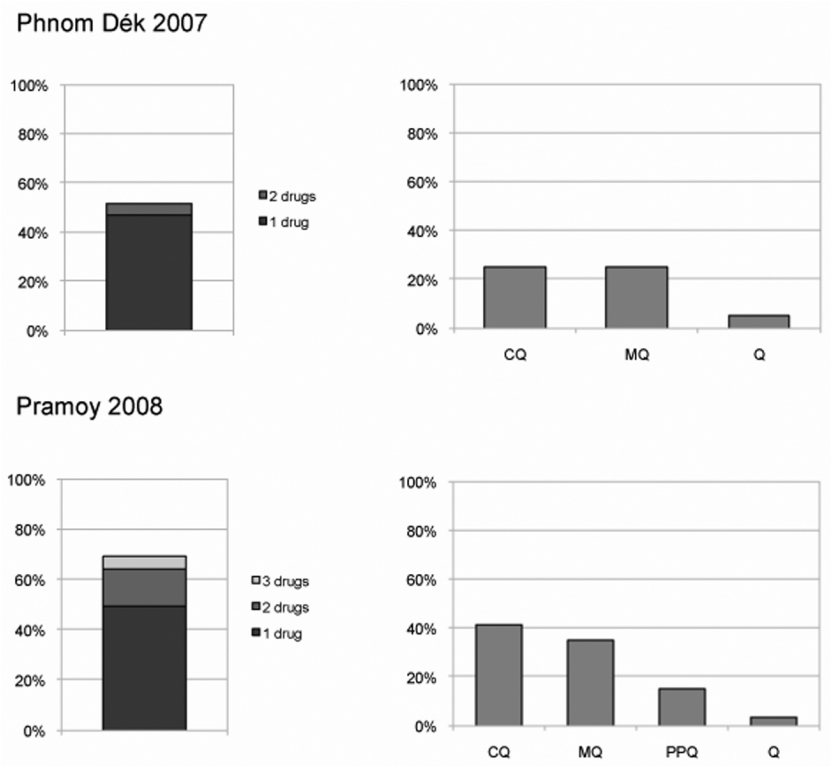

Figure 2. Percentages of patients from Preah Vihear and Pursat with residual antimalarial concentrations. CQ, chloroquine; MQ, mefloquine; PPQ, piperaquine; Q, quinine.

Table 2. Residual Plasma Concentrations of Antimalarials Found before Treatment in 61 Patients with Malaria from Pursat

Antimalarial	No. of patients	ng/mL			
		Mean	Median	Minimum	Maximum
Chloroquine	25	80.8	16.8	2.6	919.5
Mefloquine	21	431.0	346.2	2.9	1202.4
Piperaquine	9	10.7	7.9	2.1	23.9
Quinine	2	8.6	8.6	4.2	12.9

NOTE. No artemether, artesunate, dihydroartemisinin, amodiaquine, *N*-desethyl-amodiaquine, desbutyl-lumefantrine, lumefantrine, pyronaridine, sulfadoxine, or pyrimethamine was found.

sidual chloroquine, mefloquine, and piperaquine concentrations (Figure 2). For piperaquine, among the 7 eligible patients the median plasma concentration was 7.9 ng/mL (range, 6.1–22.9 ng/mL) on day 0 and was 28.5 ng/mL (range, 18.8–56.3 ng/mL) on day 14. This means that a similar dosage level should have been administered at a median of 67 days (interquartile range, 53–81 days; range, 15–93 days) before enrollment into the study, to account for the levels observed on day 0. For 1 patient (14%) this estimate was <28 days. Because there was only 1 late parasitological failure on day 28, correlation between day 0 concentration and treatment failure was not assessed. The variability in $t_{1/2}$ (lowest and highest value measured for $t_{1/2}$ [23]) translates into intervals extending from 50% to 632% of estimates (median).

DISCUSSION

To our knowledge, this is the first study to investigate the presence of a broad range of antimalarials in the plasma of Southeast Asian patients with malaria before treatment. The measurement of 14 antimalarial drugs currently in use allowed a comprehensive assessment of all circulating drugs in the studied communities in a region with high levels of antimalarial drug resistance (Pursat province) and a region with moderate levels of drug resistance (Preah Vihear province). Patients visiting a health facility represent a selected group, because they might be at a higher risk for repeated episodes of malaria (and hence treatment) due to their exposure. Such a sample was used for convenience in a pilot assessment.

Residual concentrations of former first-line treatments. Although quinine is not recommended as first-line treatment for uncomplicated falciparum malaria, it was found in 4.7% and 3.3% of patients in Phnom Dék and Pramoy, respectively. One patient had a quinine plasma concentration of 7.6 mg/L, which corresponds to plasma concentrations during acute oral treatment with ~10 mg/kg quinine every 8 h [26, 27]. Furthermore, we detected mefloquine in one-third of the study patients from Pursat, with 1 patient showing a plasma concentration above the minimum inhibitory concentration. This shows that people often buy their antimalarials from the private sector, given that in 2008 the government changed the first-line treatment along the border with Thailand from artesunate-mefloquine to dihydroartemisinin-piperaquine and thus artesunate-mefloquine was no longer available at government health facilities in western Cambodia. Effective case management, including prompt diagnosis and treatment with an appropriate antimalarial, are not guaranteed in the private sector. However, this is a key element of the global strategy to eliminate malaria on a long-term basis [28]. Comprehensive behavioral change of the population is urgently needed to encourage them to seek appropriate management (ie, laboratory diagnosis and ACT for malaria). Detection of a drug with a long residence time does not allow determination of whether a patient had taken it as monotherapy or as part of an ACT or whether the complete dose had been taken. The removal of monotherapies from the market in Cambodia must be enforced, given that people with poor access to health facilities tend to buy the cheapest antimalarial available in the private sector regardless of its efficacy against the disease.

Residual concentrations of current first-line treatments. We found that more than half of the patients carried residual antimalarials at inclusion in our study. Although chloroquine has been banned as first-line treatment for *P. falciparum* infection, it could be detected in the plasma of 25.0% of the patients in the Preah Vihear province and 41.0% of the patients in the Pursat province. This might be explained by the fact that chloroquine is the recommended first-line treatment for *P. vivax* infection in Cambodia. We also detected residual concentrations of current first-line treatments in the study patients: 26.6% with mefloquine in Preah Vihear, where the first-line treatment in 2007 was artesunate-mefloquine, and 14.7% with piperaquine in Pursat, where the first-line treatment in 2008 was dihydroartemisinin-piperaquine. Assuming that the patients had taken mefloquine (in Preah Vihear) or piperaquine (in Pursat) according to the 3-day treatment regimen, most patients must have taken the regional first-line regimen >28 days before treatment in our study. It is also possible that patients might have taken a subtherapeutic dose of these antimalarials more recently. However, as indicated by the large variability in elimination half-life, these values represent only rough estimates. The mean day 7 values observed in this study for the 7 patients with a residual dihydroartemisinin-piperaquine concentration at inclusion (mean, 49.8 ng/mL [95% CI, 30.9–68.7 ng/mL]) were comparable to those from a study of 196 patients in Papua, Indonesia (mean, 46.6 ng/mL [95% CI, 43.3–49.8 ng/mL]) [29].

Artemisinins. In our study, it was not possible to detect any of the artemisinins in the patients' plasma because of the short half-life of these compounds [30]. However, the possibility that some of the patients might have taken one of the artemisinins as monotherapy cannot be excluded. Considering the high number of patients who did consume antimalarials

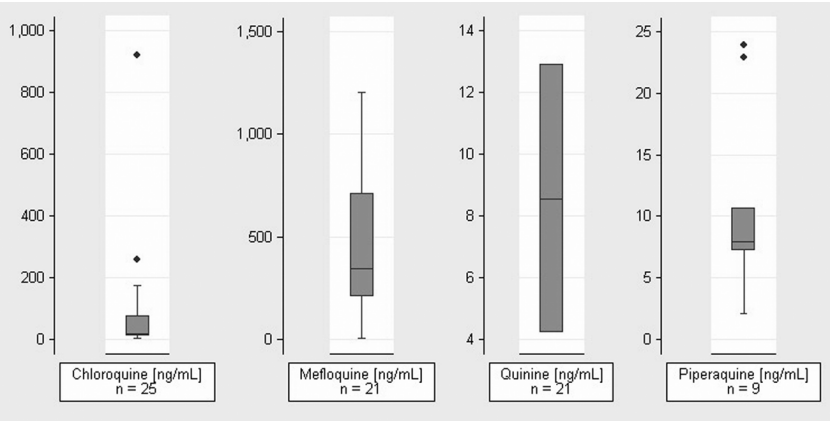

Figure 3. Residual plasma concentrations of antimalarials found before treatment in 61 patients with malaria from Pursat. The number of patients, median, 25th and 75th percentiles, lower and upper adjacent values, and outlier values are shown for chloroquine, mefloquine, quinine, and piperaquine.

that should not be used as therapy for uncomplicated falciparum malaria, it is possible that some might also have taken an artemisinin as either combination therapy or monotherapy, but this cannot be verified.

Potential bias in drug safety and efficacy assessment. Previous antimalarial intake may interfere with the outcome of the treatment under investigation. The present study shows that only baseline drug concentration measurement in the blood can be reliably used for the purpose. Our LC-MS/MS assay covers 14 antimalarials in a single run. We can confidently exclude a lack of specificity and false-positive result, because we included blank plasma samples as negative controls and systematically repeated the measurement on a new chromatographic column. Previous drug intake may affect the current treatment in several ways. Higher drug exposure resulting from cumulative levels may lead to better efficacy or more toxicity. In addition, the parasites causing the disease at the time of enrollment may be a less sensitive population selected by the previous treatment, and patients might be considered as already experiencing treatment failure at inclusion.

High drug pressure. The likelihood of selecting for drug-resistant parasites is highest with subtherapeutic levels of a single drug. Thus, it is worrying that patients with low residual concentrations of the long-lived antimalarials (mefloquine and piperaquine) present with a new episode of malaria before they have completely cleared the antimalarial. Price et al [29] reported that the mean plasma piperaquine concentration was 16.8 ng/mL (95% CI, 15.1–18.6 ng/mL) on day 28. In our study, the mean plasma piperaquine concentration on day 0 was 10.7 ng/mL. It is therefore likely that most patients had taken their piperaquine treatment as symptomatic treatment of a fever episode or as treatment of a confirmed malaria episode >1 month before inclusion. The persisting high drug pressure of mefloquine facilitates the spread of multidrug-resistant parasites from western to northern and eastern Cambodia, where parasites are still susceptible to mefloquine. The loss of artesunate-mefloquine as a treatment option for malaria in resource-poor areas such as rural Cambodia would lead to a higher burden of disease due to treatment failure. The efforts of the Ministry of Health to restrict the use of dihydroartemisinin-piperaquine only to areas of high drug resistance seems to be negated by the nonadherence of the susceptible population to bed net–use recommendations and a lack of appropriate treatment seeking behavior for first-line antimalarial combination therapy in the private and informal sector. On the other hand, the high number of patients taking antimalarial drugs on day 0 implies good drug coverage and highlights the favorable habit of using ACTs for episodes of fever even at the community level.

The present study shows that many persons still seek treatment in the private and informal sector, which does not necessarily follow national treatment policies. The emergence and spread of parasite resistance against antimalarials along the Thai-Cambodian border can be contained only via comprehensive behavioral change, communication, community-based mobilization, and advocacy. Indeed, with the help of Global Fund grants, the Ministry of Health has been encouraging easy access to first-line treatment through free delivery at all health centers as well as a large social marketing campaign in the private sector, focusing on diagnostics and providing ACTs at a low price. Recently, Cambodia was chosen to be part of the Affordable Medicines Facility–malaria initiative, an innovative financing mechanism designed to expand access to ACTs. Thus, the availability of good drugs at a low price should be sustained throughout the whole country. Currently, the Ministry of Health is extending the use of the well-tolerated dihydroartemisinin-piperaquine treatment. Adherence to this ACT is expected to be higher than that for artesunate-mefloquine, the less well-tolerated standard treatment in use since 2001. The challenge will still be to restrict the use of first-line ACTs to

confirmed malaria cases and hence ensure appropriate use of antimalarials.

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