Atovaquone-Proguanil Remains a Potential Stopgap Therapy for Multidrug-Resistant *Plasmodium falciparum* in Areas along the Thai-Cambodian Border

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Our recent report of dihydroartemisinin-piperaquine failure to treat *Plasmodium falciparum* infections in Cambodia adds new urgency to the search for alternative treatments. Despite dihydroartemisinin-piperaquine failure, and higher piperaquine 50% inhibitory concentrations (IC₅₀s) following reanalysis than those previously reported, *P. falciparum* remained sensitive to atovaquone (ATQ) *in vitro*. There were no point mutations in the *P. falciparum* cytochrome *b* ATQ resistance gene. Mefloquine, artesimisin, chloroquine, and quinine IC₅₀s remained comparable to those from other recent reports. Atovaquone-proguanil may be a useful stopgap but remains susceptible to developing resistance when used as blood-stage therapy.

The Thai-Cambodian border has long been a focus of the growing public health crisis of *Plasmodium falciparum* multidrug resistance. In 2007 to 2008, the first treatment failures with artesunate monotherapy (7 days) were reported in western Cambodia (1). We recently reported unacceptably high failure levels in northern Cambodia with the current national first-line treatment, dihydroartemisinin-piperaquine (DP), with a drop in efficacy from 90% in 2010 (2) to only 46% in 2013, associated with a 3-gene mutation in kelch-13, MAL10, and MAL13 of the *P. falciparum* genome (3). Cambodian health officials are considering alternatives to replace it as the national first-line *P. falciparum* treatment regimen.

Malarone, a fixed-dose combination of atovaquone (ATQ) and proguanil (PG), has recently been used as part of a multidrug-resistant malarial containment program in western Cambodia (4). ATQ (a coenzyme Q analogue) specifically targets the cytochrome *bc1* complex of the mitochondrial respiratory chain in the malarial parasite (5). Various single nucleotide polymorphisms (SNPs) in the quinone binding site of the *Plasmodium* species cytochrome *b* (*cytb*) gene have been implicated in conferring resistance to ATQ (6). While M133I and L271V have been implicated in murine malarial models (7, 8), and L144S, K272R, and V284F have been demonstrated in cultures exposed to high concentrations of ATQ (9), SNPs in position 268 (Y268C, Y268S, and Y268N) have been associated with clinical failure (10). ATQ resistance also appears to be associated with the delayed recrudescence of resistant parasites ≥ 3 weeks after initial clearance of parasitemia by ATQ-PG therapy (11). While likely of critical importance in the setting of rapidly worsening antimalarial resistance, little is known about the prevalence of *P. falciparum* *cytb* 268 mutations or ATQ susceptibility in Cambodia.

We isolated *P. falciparum* from blood samples collected prior to treatment from a total of 108 patients with uncomplicated *P. falciparum*, obtained during screening for a 2013 dihydroartemisinin (DHA)-piperaquine (PPQ) treatment efficacy study and diagnosed by microscopy and confirmatory *Plasmodium* species quantitative real-time PCR (12). All patients signed informed consent. All isolates were evaluated for susceptibility to a panel of

standard antimalarials, including artesunate (AS), DHA, mefloquine (MQ), quinine (QN), chloroquine (CQ), and PPQ. ATQ had not been part of the initial panel but was added after the first 21 subjects were enrolled, due to high observed PPQ failure rates. ATQ was dissolved in dimethyl sulfoxide (DMSO) and diluted in 70% ethanol and then sterile water for a final concentration range of 0.14 to 100 ng/ml, while the conditions used for other drugs were as previously described (13). Susceptibility was measured by histidine-rich protein-2 (HRP-2) enzyme-linked immunosorbent assay (ELISA) testing on fresh isolates within 4 h of phlebotomy after being incubated for 72 h in 0.5% AlbuMAX with RPMI medium on drug-coated plates, according to previously published methods (13, 14).

Figure 1 shows the *ex vivo* susceptibility results for all isolates, and the values were comparable to our other recent *ex vivo* observations for all drugs except piperaquine, which was more resistant (15). The atovaquone-susceptible *P. falciparum* W2 clone was used as an established reference (16); the highly ATQ-resistant C2B clone was also used (17). All clinical isolates and the W2 clone were sensitive to atovaquone (geometric mean 50% inhibitory concentration [IC₅₀], 6.0 nM), while the geometric mean IC₅₀ for the C2B strain was 11,368 nM (range, 9,214 to 12,242 nM) (Fig. 1).

In retrospect, 23 of 108 (22%) isolates were found to have yielded inaccurate piperaquine IC₅₀ curves following publication of the original report, as they had been capable of growing in the presence of the maximum piperaquine concentration tested

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FIG 1 Ex vivo drug susceptibility of *P. falciparum* isolates from Cambodia. IC₅₀s (in nanomoles) of *P. falciparum* mono-infection are plotted for each drug, with their geometric mean indicated by a red bar, and indicated by the value below each cluster of data points. The dashed lines denote geometric mean IC₅₀s against the *P. falciparum* W2 (green) and C2B (blue) reference clones. Red circles indicate isolates that were not originally cleared by 674 nM PPQ.

(674 nM). To better determine the IC₅₀s in these resistant isolates, we reinterpolated the IC₅₀ dose-response curves by including the optical density (OD) values of the individual patient cultures in the presence of 2,000 ng/ml CQ, the value at which 100% HRP-2 inhibition occurred. Fitting these “zero-growth” OD values for individual isolates to the previously derived piperazine curves yielded extrapolated a PPQ concentration of 53,905 nM at the point of 100% inhibition (see Fig. 2). Following this reanalysis, piperazine resistance in some clones was higher than what we had previously reported for this study, with 22 of 92 (24%) evaluable isolate IC₅₀s substantially >50 nM, the highest value seen from previous years (2009 to 2012 [15]). Further, isolates from subjects with *P. falciparum* recrudescence had a significantly increased median IC₅₀ of 40.3 nM, compared to 28.6 nM for those without *P. falciparum* recrudescence ($P < 0.05$, Mann-Whitney U test).

High-resolution melting (HRM)-PCR genotyping has been suggested as a fast and inexpensive tool for use in tracking parasite genetic polymorphisms (18). We applied HRM real-time PCR assay techniques to the samples to probe for single nucleotide polymorphisms (SNPs) associated with *P. falciparum* cytochrome *b* (*cytb*) variants at codon 268. Synthetic constructs were prepared

to scan *cytb* and develop HRM curve profiles to distinguish ATQ-resistant mutations (Tyr268Asn, Tyr268Ser, and Tyr268Cys) from the wild-type haplotype (Tyr268Tyr) using *P. falciparum* reference DNA from C2B (ATQ-resistant) and 3D7 (ATQ-susceptible) clones. While we were unable to replicate prior melting point curves or temperature ranges reported by Gan and Loh (18) using our constructs, our assay was able to recognize the controls and revealed 2 potential mutants (data not shown). Secondary analysis of the 2 flagged isolates by fluorescent peak trace chromatography revealed a heterozygous 2-base mutation at position 271, a change not known to confer ATQ resistance (data not shown). Confirmatory Sanger sequencing (19) showed all 108 isolates to be wild type, including 3 marked as indeterminate by the HRM-PCR assay, and all were sensitive to ATQ *in vitro* (Fig. 3).

While *P. falciparum* *in vitro* antimalarial resistance patterns to most currently available drugs in Cambodia were unchanged, we found worse *in vitro* resistance to piperazine than we had previously reported in isolates from a study that found high-level clinical DHA-piperazine failure. Despite this, and the recent use of atovaquone-proguanil as a part of public health containment activities in Cambodia, we found little evidence of ATQ resistance in *P. falciparum* clinical isolates from northern Cambodia. Although HRM-PCR was able to identify the 2 mutant isolates in our samples, disparities between our results and those of other published studies suggest that further evaluation of HRM-PCR is needed to

FIG 2 PPQ dose-response curves for 3 illustrative *P. falciparum* isolates that were not cleared at the maximum PPQ concentration used in the original assay. The colors denote different isolates. The solid lines are growth inhibition curves interpolated by HRP-2 OD, as measured by serial PPQ dilution with concentrations ranging from 0 to 674 nM. The dashed lines represent reanalyzed curves by including the control OD value for 100% HRP-2 inhibition (2,000 ng/ml CQ) and refitting the curve. This was equivalent to a concentration of 53,905 nM PPQ (denoted by closed circles); reinterpolated IC₅₀s using these values are denoted by vertical colored lines for each isolate.

FIG 3 DNA sequencing analysis for *cytb* mutations revealed all of the 108 isolates to be wild-type, despite 2 *P. falciparum* isolates positive for mutations in the HRM-PCR. Y268C, N and S are previously described SNP associated with atovaquone resistance. While the 3D7 *P. falciparum* clone shares the wild-type sequence, the C2B and TM90 clones share the Y268N SNP.

determine if the method is viable as a reliable alternative to genetic sequencing in tracking resistance. Atovaquone-proguanil remains highly effective for both the causal (liver-stage) prophylaxis of malaria (20) and as a blood-stage therapy. However, rapid parasitic blood-stage resistance known to occur with atovaquone (21) may quickly obviate its use both as a treatment and as one of the last remaining effective prophylactic agents. At the time of this writing, we are currently conducting a study comparing ATQ-PG in combination with oral artesunate to ATQ-PG alone (ClinicalTrials.gov registration no. NCT02297477), using a low 15-mg dose of primaquine administered on day 1. If ATQ-PG is used to replace failing first-line malarial combination therapies, such as DHA-PPQ, meticulous clinical follow-up and molecular surveillance for emerging ATQ resistance are advised.

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