Atovaquone susceptibility in Cambodia, Saunders et al.

Atovaquone-proguanil remains a potential stop-gap therapy in areas of multidrug resistant \textit{P. falciparum} on the Thai-Cambodian border

David L. Saunders\textsuperscript{1}, Suwanna Chaorattanakawee\textsuperscript{1}, Panita Gosi\textsuperscript{1}, Charlotte Lanteri\textsuperscript{1}, Somethy Sok\textsuperscript{4}, Worachet Kuntawunginn\textsuperscript{1}, Mali Ittiverakul\textsuperscript{1}, Soklyda Chann\textsuperscript{3}, Carrie Gregory\textsuperscript{1}, Char Meng Chuor\textsuperscript{2}, Satharath Prom\textsuperscript{4}, Michele D. Spring\textsuperscript{1}, Chanthap Lon\textsuperscript{3}

\textsuperscript{1} US Army Medical Component – Armed Forces Research Institute for Medical Sciences (USAMC-AFRIMS), Bangkok, Thailand

\textsuperscript{2} National Center for Parasitology, Entomology and Malaria Control, Cambodia

\textsuperscript{3} USAMC-AFRIMS, Phnom Penh, Cambodia

\textsuperscript{4} Royal Cambodian Armed Forces, Phnom Penh, Cambodia

\textsuperscript{§} - Corresponding Author, david.saunders.mil@afrims.org
Abstract

Our recent report of dihydroartemisinin-piperaquine failure in Cambodia adds new urgency to finding alternatives. Despite dihydroartemisinin-piperaquine failures, and higher piperaquine IC\textsubscript{50} following reanalysis than previously reported, \textit{P. falciparum} remained sensitive to atovaquone (ATQ) \textit{in vitro}. There were no point mutations in the \textit{P.f.} cytochrome b ATQ resistance gene. Mefloquine, artemisinin, chloroquine and quinine IC\textsubscript{50} remained comparable to prior reports. Atovaquone-proguanil may be a useful ‘stop-gap’, but remains susceptible to resistance when used as blood-stage therapy.

Report

The Thai-Cambodian border has long been a focus of the growing public health crisis of \textit{Plasmodium falciparum} multidrug resistance. In 2007-2008 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 \textit{P.f.} genome (3). Cambodian health officials are considering alternatives to replace it as the national first-line \textit{P.f.} treatment regimen.

Malarone®, a fixed dose combination of atovaquone (ATQ) and proguanil (PG), has recently been used as part of a multidrug resistant malaria containment program in western Cambodia (4). ATQ (a coenzyme Q analogue) specifically targets
the cytochrome bc1 complex of the mitochondrial respiratory chain in the malaria parasite (5). Various single nucleotide polymorphisms (SNP) in the quinine binding site of the \textit{Plasmodium spp.} cytochrome b (\textit{cytb}) gene have been implicated in conferring resistance to ATQ (6). While M133I and L271V have been implicated in murine malaria models (7, 8), and L144S, K272R, and V284F have been demonstrated in cultures exposed to high concentrations of ATQ (9), SNP in position 268 (Y268C, Y268S, Y268N) have been associated with clinical failure (10). ATQ-resistance also appears associated with delayed recrudescence of resistant parasites three weeks or more 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 \textit{P.f. cytb} 268 mutations or ATQ-susceptibility in Cambodia.

We isolated \textit{P. falciparum} from blood samples collected prior to treatment from a total of 108 patients with uncomplicated \textit{P. falciparum} screening for a 2013 DHA-PPQ treatment efficacy study diagnosed by microscopy and confirmatory \textit{Plasmodium spp.} 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), dihydroartemisinin (DHA), mefloquine (MQ), quinine (QN), chloroquine (CQ), and piperaquine (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 DMSO, diluted in 70% ethanol and then sterile water for a final concentration range from 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) ELISA testing on fresh isolates within 4 hours of phlebotomy after incubating for 72 hours in 0.5% albumax with RPMI media on drug-coated plates following published methods (13, 14).

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

In retrospect, 23 of 108 isolates (22%) 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 (674 nM). To better determine IC50 in these resistant isolates, we reinterpolated the IC50 dose-response curves by including the optical density (OD) value of the individual patient cultures in the presence of CQ at 2,000 ng/mL, the value at which 100% HRP-2 inhibition occurred. Fitting these ‘zero-growth’ OD values for individual isolates to the previously derived piperaquine curves yielded extrapolated PPQ concentrations of 53,905 nM at the point of 100% inhibition (see Figure 2). Following this reanalysis, piperaquine resistance in some clones was higher than we had previously reported for this study, with 22 of 92 (24%) evaluable isolate IC50s substantially higher than 50 nM, the highest value seen from
previous years (2009-2012 (15). Further, isolates from subjects with \textit{P. falciparum}
recrudescence had significantly increased median IC$_{50}$ of 40.3nM compared to
28.6nM for those without (P-value of Mann-Whitney U test < 0.05).

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 polymorphism (SNPs) associated with \textit{P. falciparum} cytochrome b (\textit{cytb})
variants at codon 268. Synthetic constructs were prepared to scan \textit{cytb} and develop
HRM curve profiles to distinguish ATQ-resistant mutants (Tyr268Asn, Tyr268Ser
and Tyr268Cys) from the wild type haplotype (Tyr268Tyr) using \textit{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 et al (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 chromatogram revealed a
heterozygous 2 base mutation at position 271, a substitution 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 HRM-PCR
assay, and all were sensitive to ATQ \textit{in vitro} (Figure 3).

While \textit{P. falciparum} \textit{in vitro} antimalarial resistance patterns to most currently
available drugs in Cambodia were unchanged, we found worse \textit{in vitro} resistance
than we'd previously reported to piperaquine in isolates from a study which found
high-grade clinical DHA-piperaquine failure. Despite this, and recent use of atovaquone-proguanil in this area as 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 other published studies suggest that further evaluation of HRM-PCR is needed to 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 parasite blood-stage resistance known to occur with atovaquone (21), may quickly obviate its use both as treatment and as one of the last remaining effective prophylaxis agents. At the time of writing, the authors are currently conducting a study comparing ATQ-PG in combination with oral artesunate to ATQ-PG alone (ClinicalTrials.gov NCT02297477), with a low 15mg dose of primaquine administered on day 1. If ATQ-PG is used to replace failing first-line malaria combination therapies such as DHA-PPQ, meticulous clinical follow up and molecular surveillance for emerging ATQ resistance are advised.

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References


using a reference clone to improve comparisons of Plasmodium falciparum field isolates.

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**Figure 1.** Ex vivo drug susceptibility of *P. falciparum* isolates from Cambodia. IC$_{50}$s (nM) of mono *P. falciparum* 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. Dashed lines denote geometric mean IC$_{50}$s against the *P. falciparum* W2 (green), and C2B (blue) reference clones. Red circles indicate isolates that were not originally cleared by 674nM PPQ.

**Figure 2.** PPQ Dose response curves for 3 illustrative *P. falciparum* isolates that could not be cleared at the maximum PPQ concentration used in the original assay. Colors denote different isolates. Solid lines are growth inhibition curves interpolated by HRP2 OD measured by serial PPQ dilution with concentrations ranging from 0-674 nM. Dashed lines represent reanalyzed curves by including the control OD value for 100% HRP-2 inhibition (CQ 2,000 ng/mL), and refitting the curve. This was equivalent to a concentration of 53,905 nM PPQ (denoted by solid circles) – re-interpolated IC50s using these values are denoted by vertical colored lines for each isolate.

**Figure 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.f. clone shares the wildtype sequence, the C2B and TM90 clones share the Y268N SNP.