INTRODUCTION

In Southeast Asia, the high prevalence of multidrug-resistant malaria has led to the implementation of combination therapies, including artemisinin-based regimens, as the most effective strategy to treat *Plasmodium falciparum* malaria while minimizing the emergence of drug resistance.1 Artesunate-mefloquine is widely recommended as a treatment combination in Southeast Asia; however, its cost and tolerability profile remain obstacles to widespread use. Furthermore, artemisinin-based combinations are not currently available to treat drug-resistant malaria in North America.

In addition to artemisinin-based combinations, a fixed combination of atovaquone-proguanil (Malarone®; GlaxoSmithKline, Research Triangle Park, NC), has previously been shown to be effective in the treatment of drug-resistant *P. falciparum* malaria with cure rates significantly higher than amodiaquine,2 mefloquine,3 chloroquine, and sulfadoxine/pyrimethamine.4 The mechanism of action of atovaquone is by inhibition of parasite mitochondrial electron transport at the level of the cytochrome bc1 complex and collapse of mitochondrial membrane potential.5,6 Proguanil inhibits plasmodial dihydrofolate reductase (DHFR) primarily through its metabolite cycloguanil, which results in disruption of parasite DNA synthesis.7 Both in vivo and in vitro studies have demonstrated synergistic anti-malarial action of atovaquone and proguanil, which results in high cure rates in patients with drug-resistant malaria, even when infections are caused by parasites containing mutations in DHFR known to confer resistance to cycloguanil.8,9 The mechanism of synergy of proguanil with atovaquone is postulated to be by its biguanide mode of action rather than through its cycloguanil metabolite.9 Recent studies have reported several cases of atovaquone-proguanil treatment failure in which resistance was linked to a single amino acid mutation at position 268 of cytochrome b (cyt b), resulting in a tyrosine to serine10,15 or tyrosine to asparagine substitution.16 In addition to these cases, a small number of treatment failures have been reported with parasites possessing wild-type cyt b. However, to date no alternative molecular mechanisms of resistance have been defined.17,18

Atovaquone-proguanil is currently a first-line agent for the treatment of *P. falciparum* malaria. However, there is a paucity of recent data examining the efficacy of atovaquone-proguanil in the treatment of multidrug-resistant malaria. This paucity, combined with an increasing number of case reports of atovaquone-proguanil treatment failure, indicate that continued monitoring of the effectiveness of this drug combination will be essential to detect the emergence and spread of atovaquone-proguanil resistance. We conducted an open-label trial on the Thailand-Myanmar border to examine the efficacy of atovaquone-proguanil in an area of multidrug resistance and herein report an overall cure rate of 98%.

METHODS AND MATERIALS

Study design. This single-center, open-label trial was conducted between November 2004 and December 2005 at the Hospital for Tropical Diseases at Mahidol University in Bangkok, Thailand. The study was reviewed and approved by the Ethics Committee of Mahidol University.

Study population. Sequential patients with acute, uncomplicated *P. falciparum* malaria were screened for inclusion in the study. Patients were eligible if they were more than 14 years of age, had a microscopically confirmed *P. falciparum* infection, had a urine test result negative for chloroquine or sulfonamides, were able to tolerate oral therapy, and were able to give informed consent. Patients were excluded if they fulfilled any one of the World Health Organization criteria for severe or complicated malaria, had an allergy to atovaquone or proguanil, had a history of anti-malarial drug ad-
ministration within two weeks of presentation, or were pregnant or breast-feeding.

**Procedures.** One hundred forty patients fulfilled eligibility criteria and after providing informed consent were enrolled in the study. Patients were administered a fixed dose of atovaquone (1,000 mg/day) plus proguanil (400 mg/day) once a day with food for three days. Study drug was provided as directly observed therapy by the site investigators. Participants were hospitalized for the duration of treatment and asked to remain in Bangkok, an area of non-transmission of malaria, for the duration of the 28-day follow-up period.

Giemsastained thick and thin blood films were prepared every six hours for quantification of parasitemia until participants were malaria smear negative. Thereafter, thick and thin blood films were taken weekly beginning on day 7 until day 28 and/or with any recurrent fever or symptoms compatible with malaria. Parasitemia was quantified per 1,000 red blood cells on a thin film or per 200 white blood cells on a thick film and expressed as parasites per microliter. A slide was considered negative after examination of 200 oil-immersion fields. Oral temperature was recorded every four hours until patients were afebrile (temperature < 37.5°C) for at least 24 hours. After enrollment, participants were examined daily for clinical signs of malaria. Thereafter, participants were examined weekly on days 7, 14, 21, and 28.

**Outcome.** The primary endpoint was the 28-day cure rate. The response to treatment was based on the World Health Organization (WHO) classification system. A sensitive response (S) is indicated by parasite clearance within seven days with no recrudescence during the 28-day follow up period.

Resistant responses (RI–RIII) were categorized according to the WHO criteria for 4-aminoquinolines: RI = parasite clearance within seven days, followed by recrudescence within 28 days; RII = reduction of parasitemia to < 25% of initial parasite count at 48 hours, but without total clearance within a 7-day period; RIII = parasitemia ≥ 25% of initial parasite count at 48 hours after initiation of therapy.

**Data analysis.** Descriptive statistics were calculated for continuous variables. Geometric means were calculated for initial parasitemia. Participants who failed to complete 28 days of follow-up were excluded from the efficacy analysis.

Cure rates were calculated from the ratio of S response/total of S + RI + RII + RIII, and 95% confidence intervals (CIs) were calculated. Parasite clearance times (PCTs) and fever clearance times (FCTs) were calculated for efficacy analysis.

Mean PCTs were calculated from initiation of anti-malarial treatment until the blood films were negative for *P. falciparum* asexual stages. Similarly, FCTs were calculated from the commencement of therapy until the body temperature decreased to < 37.5°C and the patient remained afebrile for at least 24 hours.

**Recrudescent infection.** For patients with recurrent parasitemia, genomic DNA was extracted from whole blood samples taken on the day of presentation (pre-treatment) and the day of recurrence using Qiagen (Valencia, CA) columns. To distinguish clinical failure from re-infection, a gene encoding merozoite surface protein-1 was amplified by a polymerase chain reaction (PCR) and subjected to genotyping using single-strand conformational polymorphism analysis, as described. In addition, the cyt b gene was amplified by PCR and sequenced to detect mutations that have been linked to resistance to atovaquone-proguanil *in vivo.*

### RESULTS

**Patient characteristics.** One hundred forty patients with acute uncomplicated *P. falciparum* malaria were enrolled during the study period. Participants had a mean of less than two documented episodes of malaria within the preceding two years. Three patients dropped out of the study between day 15 and day 24 because of personal social reasons. The age range of the patients was 14–56 years, and the male:female ratio was 7:1 (Table 1). The pre-malaria health status of all patients was considered good.

**Efficacy.** At enrollment, the mean initial parasitemia was 11,217.7 parasites/μL (range = 15–192,000 parasites/μL) (Table 1). At the 28-day follow-up, 137 evaluable patients had an overall cure rate of 97.8% (95% CI = 95.4–100.0%) (Table 2). Mean PCT was 41.9 hours (range = 4–92 hours), and the mean FCT was 37.1 hours (range = 4–158 hours) (Table 2).

Three treatment failures occurred during the trial: 1 RIII pattern, and 2 RI patterns, both of whom failed on day 28. These patients were successfully treated with an artemisinin plus mefloquine combination. The PCR and genotyping analysis on the two RI isolates from the day of presentation and the day of recrudescence showed that these two patients had recrudescent infection and were therefore categorized as treatment failures. The risk of re-infection in any participant had been considered very low because Bangkok is an area of non-transmission of malaria.

Sequencing of the cyt b gene of isolates from the day of presentation and day of treatment failures from all three patients who failed atovaquone-proguanil therapy showed that all isolates at time of presentation and at time of recrudescence contained wild-type sequence at position 268 and at position 133 of cyt b.

### DISCUSSION

Clinical trials largely performed in the 1990s showed that atovaquone-proguanil was highly effective for the treatment of uncomplicated *P. falciparum* malaria, with cure rates between 87% and 100%. In Thailand, atovaquone-proguanil had previously shown cure rates of 100% and 97.2%. However, recent data on the efficacy of atovaquone-proguanil for the treatment of multidrug-resistant *P. falciparum* malaria are lacking. With increasing reports of atovaquone-proguanil treatment failure and increasing prophylactic use in areas of multidrug resistance, we sought to investigate the current efficacy of this drug along the Thailand-Myanmar border, where multidrug-resistant *P. falciparum* malaria is common. We report that a fixed dose of atovaquone-proguanil once a day for three days remains effective.

#### Table 1

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<th>Table 1: Patient characteristics at the time of admission</th>
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<tbody>
<tr>
<td>Mean ± SD age, years (range)</td>
<td>26.3 ± 9.9 (14–56)</td>
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<tr>
<td>Sex ratio (M:F)</td>
<td>7:1</td>
</tr>
<tr>
<td>No. with <em>Plasmodium falciparum</em></td>
<td>140</td>
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<tr>
<td>Parasitemia, parasites/μL</td>
<td></td>
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<tr>
<td>Mean ± SD</td>
<td>11,217 ± 27,470.5</td>
</tr>
<tr>
<td>Median</td>
<td>270.5</td>
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<tr>
<td>Range</td>
<td>15–19,200</td>
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highly efficacious for the treatment of acute uncomplicated *P. falciparum* malaria, with a cure rate of 97.8% (95% CI = 95.4–100.0%).

Previous studies reported that the mean PCTs in patients treated with atovaquone-proguanil ranged from 46 to 72 hours.\(^{4,22–25}\) Consistent with these observations, we observed a mean PCT of 41.9 hours. Similarly, the mean FCT was 37.1 hours, which is well within the range reported previously.\(^{5,22–25}\) Together, these data suggest that atovaquone-proguanil remains an effective drug 10 years after its introduction for the treatment of multidrug-resistant *P. falciparum* malaria acquired in Southeast Asia.

In this trial, two patients with recrudescence parasitemia were identified as atovaquone-proguanil treatment failures, as well as one case of RIII resistance. Previous studies have linked atovaquone-proguanil treatment failure with mutations at position 268 (Tyr268Asn or Tyr268Ser) of plasmodial cyt b.\(^{10–16}\) However, several other reported cases have failed to establish an association between atovaquone-proguanil treatment failure and cyt b mutations.\(^{17,18}\) Consistent with these latter reports, we found no mutations at position 268 of cyt b in parasite isolates from patients with recrudescence parasitemia or RIII resistance in this study. In addition, we did not find mutations at position 133 of cyt b, which has been associated with atovaquone resistance in *vitro*.\(^{21}\) Collectively, these observations suggest that other mechanisms were involved in these treatment failures.

<table>
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<th>Efficacy of 28-day follow-up*</th>
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<tr>
<td>Total no. of patients</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Total no. evaluable patients</td>
<td>137</td>
<td></td>
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<tr>
<td>No. (%) of patients cured</td>
<td>134 (97%) (95% CI = 95.4–100.0%)</td>
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<tr>
<td>Parasite clearance time (hours)†</td>
<td>41.9 ± 22.1 (95% CI = 38.3–45.6)</td>
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<tr>
<td>Median</td>
<td>37</td>
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<tr>
<td>Range</td>
<td>4–92</td>
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<tr>
<td>Fever clearance time (hours)‡</td>
<td>37.1 ± 37.4 (95% CI = 30.8–43.4)</td>
<td></td>
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<tr>
<td>Median</td>
<td>26</td>
<td></td>
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<tr>
<td>Range</td>
<td>4–158</td>
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</table>

\(*\) CI = confidence interval.

† Parasite clearance time was analyzed in 139 patients.

‡ Fever clearance time was analyzed in 136 patients.

There are a number of potential explanations accounting for treatment failure in these cases. They include failure to initiate or complete therapy; vomiting, diarrhea, or other gastrointestinal conditions resulting in inadequate absorption of active drug components; or a novel molecular basis of resistance including mutations at other sites in cyt b or in other putative drug targets. Therapy in this trial was directly observed; however, drug levels were not determined and we cannot eliminate the possibility of inadequate absorption. Mutations at alternative sites on the cyt b gene or in an as, yet, unidentified new target remain a possibility. A number of mutations in cyt b, in addition to those at position 268 and 133, have been reported in association with in *vivo* resistance to atovaquone. However, none of these have been demonstrated in clinical isolates associated with in *vivo* treatment failure. Collectively, the data suggest that cyt b mutations at position 268 are sufficient, but may not be necessary, for atovaquone-proguanil treatment failure. However, further studies exploring alternative molecular mechanisms of resistance to atovaquone-proguanil are required to confirm or refute this hypothesis.

In summary, 97.8% of patients enrolled in this study were cured by a standard three-day course of atovaquone-proguanil therapy. Our data show that atovaquone-proguanil is still efficacious in the treatment of uncomplicated *P. falciparum* malaria acquired in a region where multidrug-resistant isolates are prevalent. Continued surveillance for the emergence and spread of atovaquone-proguanil–resistant malaria will be essential to prolong the lifespan of this combination agent.

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