PROPHYLACTIC ACTIVITY OFATOVAQUONE AGAINST PLASMODIUM FALCIPARUM IN HUMANS

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Abstract. The prophylactic antimalarial activity of atovaquone was determined in a randomized, double-blind, placebo-controlled study of healthy volunteers who were challenged by the bite of Plasmodium falciparum-infected Anopheles stephensi. Subjects were randomly assigned to one of three groups: six received seven daily doses of 750 mg of atovaquone, starting the day before challenge; six received a single dose of 250 mg of atovaquone the day before challenge; and four received placebo. Polymerase chain reaction-and culture-confirmed parasitemia developed in all four placebo recipients, but in none of the drug recipients, indicating that either of the atovaquone regimens provides effective prophylaxis (P = 0.005). However, in low-dose recipients, the drug levels by day 6.5 were profoundly subtherapeutic, indicating that parasites were eliminated prior to the establishment of erythrocytic infection. Atovaquone thus protects non-immune subjects against mosquito-transmitted falciparum malaria, and has causal prophylactic activity.

The increasing problem of drug-resistant Plasmodium falciparum has drastically compromised the utility of existing antimalarial drugs and no vaccine is available. This situation poses a threat not only to those living in endemic areas, but also to the millions of non-immune travelers who visit malaria countries every year. The need for safe and effective new drugs, for both treatment and prophylaxis of falciparum malaria, is well-recognized to be acute. Atovaquone is a hydroxynaphthoquinone that has novel molecular mechanisms of action in erythrocytic parasites: selective inhibition of mitochondrial electron transport, diminution of pyrimidine biosynthesis, and collapse of mitochondrial membrane potential. Atovaquone kills erythrocytic P. falciparum, including multidrug-resistant strains, in vitro and in primates. However, results of clinical trials of atovaquone against erythrocytic infections were disappointing. In an early study, five of five non-immune travelers with established erythrocytic P. falciparum infection recrudesced after treatment with 500 mg of atovaquone. Recrudescent parasites remained drug-sensitive, indicating that the plasma levels achieved were insufficient to eradicate erythrocytic stages. In subsequent field trials, failure rates of 30% were attributed to atovaquone resistance. Based on these findings, atovaquone alone is no longer being developed for clinical use against malaria. Instead it is now formulated in fixed combination with proguanil, with which it is synergistic (Malarone®; Glaxo Wellcome, Inc., Research Triangle Park, NC).

Ample clinical evidence indicates that atovaquone and proguanil, individually or in combination, act against erythrocytic falciparum parasites, and that proguanil alone has activity against exoerythrocytic stages. Although atovaquone affects P. berghei in cultured hepatocytes and in rats, no study has addressed the question of whether atovaquone itself has causal activity against P. falciparum. This issue is of considerable interest for several reasons. On a molecular basis, action against both hepatic and erythrocytic stages would suggest that atovaquone (unlike most other antimalarial drugs) targets a mechanism that is vulnerable in both life cycle stages, providing a valuable clue for future drug development. From a therapeutic perspective, proguanil has a short duration of action and widespread resistance already exists. If Malarone is to provide reliable causal prophylaxis, atovaquone must add to (or synergize with) the action of proguanil against exoerythrocytic parasites.

This study was designed to provide a stringent test of the prophylactic activity of atovaquone against falciparum malaria in humans by challenging non-immune subjects with heavily infected mosquitoes. It was devised to mimic the risk of malaria encountered by travelers, yet to exclude variables that confound the interpretation of prophylaxis studies conducted in an endemic area (e.g., varying degrees of immunity, infections not initiated at a single point in time). Within this context, it was further designed specifically to detect activity against exoerythrocytic parasites. The results indicate that atovaquone protects non-immune subjects against mosquito-borne falciparum malaria, and that this protection has a causal component.

METHODS

This was a randomized, double-blinded, placebo-controlled, outpatient study, conducted under a physician-sponsored Investigational New Drug Application with approval of the Johns Hopkins Joint Committee on Clinical Investigation (our Institutional Review Board). Subjects were dosed, challenged, and followed as a single cohort in the National Institutes of Health (Bethesda, MD)—sponsored outpatient General Clinical Research Center of the Johns Hopkins Hospital. The day of challenge is designated study day 0; patency is defined as the detection by microscopy of parasites in blood.

Subjects. Healthy volunteers were sought by advertisements and were eligible if they were 18–45 years old, within 25% of ideal weight, able to score 80% correct on a questionnaire to test comprehension of malaria and the study, able to provide a responsible person to assist with follow-up, and if they had blood of type A or O that supported infection recrudesced P. falciparum asites in blood.

The purpose of the study was to evaluate the prophylactic activity of atovaquone against Plasmodium falciparum in non-immune subjects.
allergy to mosquito bites or intolerance of antimalarial drugs; or use of any anti-infective drug or quinine-containing drink in the week preceding atovaquone dosing. Written informed consent was obtained for the screening process, for human immunodeficiency virus testing (to rule out infection of healthy volunteers), and for the study.

The formulas of Lachin\textsuperscript{22} were applied to obtain a sample size estimate of 10, assuming $\alpha = 0.05$, $\beta = 0.2$, 100% infection in the placebo group, and 20% infection in a treatment group (we chose to compare each drug treatment with placebo, but not to compare the two drug treatments). With these criteria, a placebo:drug ratio of 2:3 does not alter the required number of 10 subjects; thus, there were four placebo recipients and six in each of two treatment groups, for a total of 16 volunteers. This number is small enough for a safe and efficient study, yet large enough to provide an estimate of efficacy that has an exact binomial 95% confidence interval of 61–100% (if $6 \text{ or } 6$ in a treatment arm are protected). To ensure availability of 16 volunteers for challenge, the volunteers were selected for challenge. To minimize the biological variation inherent in a sporozoite challenge, the volunteers were studied in a single cohort.

**Drug administration and randomization.** Atovaquone (250 mg tablets) and matching placebo were provided by Glaxo Wellcome. Three regimens were evaluated; in each of these, daily doses were administered orally under direct supervision for seven days, starting day −1. The high-dose group received three 250 mg tablets of atovaquone (750 mg) daily; the low-dose group received one tablet of atovaquone (250 mg) and two matching placebo tablets on day −1, then three placebo tablets daily; the placebo group received three placebo tablets daily. Volunteers were asked to fast after midnight prior to, and for two hours after, dosing. On day −1, drug or placebo was given with a breakfast containing 0.7 grams of fat, and on days 0–5, with 11.1 grams of fat to maximize absorption.\textsuperscript{9}

**Mosquitoes, parasites, and challenge.** *Anopheles stephensi* were propagated and maintained in an environmentally-controlled insectary in the Johns Hopkins School of Hygiene and Public Health. About 400 three-day-old mosquitoes were infected by standard methods\textsuperscript{23} with 16–18-day-old gametocyte cultures of the NF54 strain of *P. falciparum*.\textsuperscript{24} Chloroquine and atovaquone sensitivities were confirmed prior to infection (50% effective concentrations $[EC_{50}] = 2.7$ and 0.19 ng/ml, respectively).\textsuperscript{25} Sixteen days after infection each volunteer was bitten by five infected mosquitoes as described previously.\textsuperscript{23} Thereafter, each subject was given a pager and wallet card, and was fitted with a medical alert bracelet.

**Measures of efficacy and safety.** Follow-up visits were scheduled daily (days 5–21), every other day (days 22–35), then weekly until 12 weeks after challenge. Volunteers returned one year after challenge for final evaluation and to have the bracelets removed. At each visit, subjects were interviewed briefly in a nondirected fashion, temperature was measured, and blood was drawn for a parasitologic examination: a quantitative buffy coat (QBC) tube\textsuperscript{26} and Giemsa-stained thick and thin smears at every visit; polymerase chain reaction (PCR) until day 21; and cultures (maintained in vitro for 30 days) on study days 7 and 8. The QBCs and thick smears were evaluated at once; PCR samples were analyzed after week 12. Blood was drawn for routine laboratory tests at one, two, and three weeks after dosing. Before unblinding (which occurred seven months after challenge), all symptoms, signs, or laboratory findings had been evaluated for severity and judged for relation to atovaquone, malaria, and/or the study. On days 4 and 7, urine was collected to assay for other antimalarial drugs.\textsuperscript{27}

All positive results in microscopic analyses were confirmed by two experienced observers; a positive QBC tube result was considered sufficient to initiate chloroquine therapy. When parasitemia was first detected, blood was drawn for safety monitoring, PCR, parasite culture and sensitivity, and drug levels. Oral chloroquine phosphate was then administered promptly: 1 gram followed by 500 mg at 6, 24, and 48 hr. Volunteers were monitored daily until negative for three consecutive days and symptom-free. Blood for safety monitoring was taken when indicated during illness, at the time of the third negative smear, and six weeks thereafter. For the last three subjects who developed parasitemia (#7, 8, and 13), resolution was assayed by PCR on at least three occasions after starting treatment with chloroquine.

**Pharmacokinetics.** Blood for atovaquone levels was drawn before dosing, on days −1, 0, 1, 3, and 5, and on days 7, 9, 11, 14, 17, 21, 29 and 49. Plasma was collected and stored at −80°C for high-performance liquid chromatography analysis at Glaxo Wellcome. Elimination half-lives were determined by linear regression of log plasma concentration-time curves; areas under the plasma concentration-time curve (AUCs) were calculated by the linear trapezoidal rule. For subjects #1, 4, 6, and 19, whose plasma concentrations decreased below the limit of detection during the 6.5–11.5-day interval, the concentration at 11.5 days was extrapolated from the last available point, using a 3.76 day half-life (mean for 10 evaluable subjects, Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Kinetic parameter*</th>
<th>AUC\textsubscript{0−6}</th>
<th>AUC\textsubscript{0−+12}</th>
<th>T\textsubscript{1/2}</th>
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</thead>
<tbody>
<tr>
<td><strong>High dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>18.7</td>
<td>10.6</td>
<td>3.8</td>
</tr>
<tr>
<td>#3</td>
<td>8.51</td>
<td>10.4</td>
<td>7.4</td>
</tr>
<tr>
<td>#5</td>
<td>18.4</td>
<td>11.0</td>
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</tr>
<tr>
<td>#10</td>
<td>24.1</td>
<td>16.6</td>
<td>4.5</td>
</tr>
<tr>
<td>#15</td>
<td>29.5</td>
<td>19.5</td>
<td>2.6</td>
</tr>
<tr>
<td>#16</td>
<td>21.4</td>
<td>15.4</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>20.1 ± 7.0</td>
<td>13.9 ± 3.8</td>
<td>3.9 ± 1.9</td>
</tr>
<tr>
<td><strong>Low dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>1.77</td>
<td>0.48</td>
<td>NC</td>
</tr>
<tr>
<td>#4</td>
<td>0.56</td>
<td>0.33</td>
<td>NC</td>
</tr>
<tr>
<td>#6</td>
<td>1.67</td>
<td>0.31</td>
<td>3.1</td>
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<tr>
<td>#19</td>
<td>2.13</td>
<td>0.47</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>2.39 ± 1.55</td>
<td>0.72 ± 0.52</td>
<td>3.6 ± 0.6</td>
</tr>
</tbody>
</table>

* AUC = area under the plasma concentration-time curve; NC = not calculated because plasma levels fell below the limit of detection.
Polymerase chain reaction. Blood was collected in sodium citrate and stored at ~80°C. Whole blood (200 μl) was lysed with an equal volume of 10 mM Tris-HCl (pH 8.0), 75 mM EDTA, 1% sodium dodecyl sulfate, 1.2 mg/ml of proteinase K. The lysate was incubated at 55°C for 60 min, then at 37°C for 30 min with 100 μg/ml of RNase A and 200 units/ml of RNase T1. Digests were extracted twice with phenol, and the DNA was precipitated with ethanol and dissolved in 66 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The DNA in 20-μl aliquots was amplified in nested PCRs using primers and conditions described previously. All samples were assayed at least twice. In our hands this method detects two parasites/ml of blood, consistent with previous reports and roughly equivalent to the sensitivity of in vitro culture. If just one parasite injected by a mosquito generates 30,000 in the liver, then the initial parasitemia should be ~6/ml, a level readily detectable by our PCR and culture methods.

RESULTS

Study population. Of 183 candidates who responded to the advertisements, 152 failed the telephone interview, seven failed laboratory tests, one was overweight, three chose not to continue, and one failed the malaria questionnaire; the remaining 19 were enrolled and 16 were challenged. Of those 16, the average age was 30.1 years (range 5–22±44); there was one woman, and one Hispanic, four African-American, and 11 white subjects. There was no significant difference among the high-dose, low-dose, or placebo groups in average age (28.8, 31.8, and 29.5 years, respectively) or race. Volunteers 10 and 17 were identical twins. Of the 654 scheduled outpatient visits (including the one-year follow-up visit), three were missed (99.5% overall compliance), all by a single volunteer for whom the frequent visits posed a hardship. He was therefore admitted to the inpatient research unit for seven weeks of the study.

Challenge. Challenge of all 16 subjects was completed in 3 hr, starting 1 hr after the second dose of drug/placebo (25–28 hr after low-dose atovaquone). The mosquitoes were uniformly and heavily infected: only 89 mosquitoes were required to obtain 80 that were infected (90% rate) and in these 80 the salivary gland sporozoite index was 3.3 ± 0.8 (mean ± SD). There were no significant differences among high-dose, low-dose, or placebo groups in the average number of mosquitoes required to obtain five infected bites (5.5, 5.8, or 5.3, respectively; range = 5–8) or the mean sporozoite index (3.5, 3.2, or 3.4, respectively; range = 2.8–3.8).

Efficacy. Four subjects, all placebo recipients, developed microscopic parasitemia on days 9, 13 (two subjects), or 18 after challenge (Figure 1). The salivary gland indices for mosquitoes infecting these volunteers were 3.6, 3.0, 3.0, and 3.8, respectively. When parasitemia was first detected, three subjects were symptomatic (see below); subject 8 was entirely asymptomatic. All four positive individuals were confirmed by culture and PCR. The latter detected parasites at least two, and up to six, days before they were evident by microscopy (Figure 1). Consistent with a 6.5 day minimum pre-erythrocytic period for the NF54 strain of P. falciparum, circulating parasites did not appear prior to day 7. Parasites were not detectable by any method in subject 7 on day 8, perhaps a result of sequestration. Volunteers 7 and 17, who were positive in the last scheduled PCR determi-
nation, were PCR negative four months later (late, Figure 1). Parasites obtained at patency remained atovaquone-sensitive (EC50 = 0.17–0.33 ng/ml).

Based on the thick smear and QBC tubes, none of the 12 drug recipients developed microscopic parasitemia; these negative results were confirmed by PCR and culture (Figure 1). No volunteer had evidence for surreptitious use of drugs with antimalarial activity.

**Safety and tolerance.** At the time parasitemia was first detected, three subjects had headache, myalgias, chills, sweats, and fever (37.9°C or less), minimal aminotransferase elevations (≥ 1.09 upper normal), thrombocytopenia (2 subjects; ≥ 0.97 lower normal), leukopenia (1 subject; 0.79 lower normal), and proteinuria (1 subject, trace). All findings returned to normal levels after chloroquine treatment, although symptoms worsened on the first day of treatment, as noted previously.6

Events judged to be mild and possibly drug-related were seen in six of six high-dose, five of six low-dose, and four of four placebo recipients. In only one subject, a high-dose recipient, were nausea and gastrointestinal upset considered moderate and likely to be drug-related. Six volunteers became mildly anemic (≥ 0.92 lower normal), which was likely due to repeated blood sampling. All findings disappeared or reverted to normal levels on repeat evaluation.

**Pharmacokinetics.** Plasma levels at 24 hr after the first dose of atovaquone were comparable to those reported previously when the tablet formulation was administered with a no-fat meal.9 Terminal half-lives (overall mean = 3.8 days or 90 hr) were somewhat longer than the expected 70 hr (Table 1). Plasma levels compiled in Table 1 and depicted in Figure 2 are divided to correspond with the concurrent stage of infection: days 0–6.5, to reflect causal activity during the period of hepatic infection; and days 6.5–11.5, to reflect suppressive activity during the earliest possible five days of erythrocytic infection. The choice of day 6.5 for first appearance of circulating parasites was based on careful clinical studies of the NF54 strain of *P. falciparum,8* which we used in this study. A five-day AUC during the erythrocytic phase was chosen to relate our data to previous results against erythrocytic parasites in non-immune individuals.10 In that report, the mean ± SD AUC in the first five days after treatment with a single oral dose of 500 mg of atovaquone was 5.6 ± 2.9 μg·day/ml (range = 3.7–10.4).

**DISCUSSION**

We found that atovaquone protects non-immune subjects bitten by mosquitoes heavily infected with *P. falciparum,* and all evidence indicates there is activity against exoerythrocytic parasites.

The high-dose regimen was designed to evaluate atovaquone at the maximum dose practical for prophylaxis, thus to preclude the necessity for further studies if it should fail. All six volunteers in the high dose regimen were protected, in contrast to all four placebo recipients who developed malaria (P = 0.005, by two-tailed Fisher’s exact t-test; 95% confidence interval of protection = 61–100%). Although this result provides clear evidence for prophylactic activity, it leaves some uncertainty about the mechanism by which prophylaxis was achieved. Highly sensitive methods failed to detect any circulating parasites (Figure 1), strongly suggesting that the antiparasitic effect occurred during the first 6.5 days of the life cycle of the parasite. However, at day 6.5, residual plasma atovaquone levels may have been sufficient to affect erythrocytic stages (Table 1 and Figure 2), leaving open the unlikely but still possible contribution of suppressive activity.

To identify unambiguously the mechanism of prophylaxis, the low-dose regimen was designed so that atovaquone would peak during the hepatic stages of the life cycle, then decrease below therapeutic levels by day 6.5, when parasites are first expected to appear in circulating red blood cells. This goal was successfully achieved: for low-dose recipients the mean AUC5.5–11.5 was eight-fold less than that which uniformly failed against erythrocytic infections in non-immune travelers (Table 1 and Chiodini and others10; P < 0.003, by two-tailed t-test). All low-dose recipients in our study had AUC5.5–11.5 values at least two-fold below the lowest level that failed previously, and the values ranged as much as 34-fold below the highest level that failed. Although 6.5 days was chosen as the theoretical start point for erythrocytic infection, the experimental results are not substantially different if the AUC is taken from day 5.5 (the mean ± SD AUC5.5–11.5 is 0.85 ± 0.61 μg · day/ml), the earliest appearance reported for any strain of *P. falciparum.*12 Indeed, for some of our low-dose subjects, AUC values calculated from day 6.5 may overestimate the amount of drug present during earliest parasitemia: two placebo recipients had no detectable circulating parasites until 10 or 16 days after challenge (Figure 1).

The low-dose arm of the study provides three lines of
evidence that atovaquone acts against hepatic stages of the life cycle of the parasite. First, all six subjects were protected, compared with placebo recipients ($P = 0.005$, by two-tailed Fisher's exact $t$-test; 95% confidence interval of protection $= 61–100\%$), despite drug levels by day 6.5 that were clearly subtherapeutic for erythrocytic stages. Second, two highly sensitive methods did not detect subclinical parasitemia (Figure 1), a possible outcome if causal prophylaxis had failed and erythrocytic parasites were eliminated before becoming apparent by microscopy. (In placebo recipients, parasites were detected up to six days before microscopic patentcy.) Third, we monitored for one year after challenge, ruling out a prolonged hepatic phase seen when parasites are simply delayed or inhibited in partial response to vaccines or drugs.

The results thus support the conclusion that atovaquone destroys exoerythrocytic \textit{P. falciparum} in humans, consistent with its reported activity against rodent malaria \textit{in vitro} and in animals.\textsuperscript{18,19} Plasma levels during the first 6.5 days of treatment with its reported activity against rodent malaria \textit{in vitro} it is possible that differences in mitochondrial function might render the hepatic stages more susceptible to this drug.

Our study indicates that atovaquone can protect non-immune subjects against mosquito-transmitted \textit{P. falciparum}, even when given the day before exposure and under conditions that minimize its absorption (administration with a very low fat meal). If so, travelers need not continue prophylaxis after leaving an endemic area, a distinct advantage over the current suppressive regimens that require four weeks of treatment after exposure. This important practical consideration certainly merits confirmation in larger trials with non-immune subjects exposed to an array of \textit{P. falciparum} strains in various parts of the world. Although atovaquone alone was effective in this study, the ready selection of drug-resistant parasites dictates that for malaria it should be administered only in combination with proguanil.

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