Transmissibility of clinically relevant atovaquone-resistant *Plasmodium falciparum* by anopheline mosquitoes

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Abstract

Rising numbers of malaria cases and deaths underscore the need for new interventions. Long-acting injectable medications, such as those now in use for HIV prophylaxis, offer the prospect of a malaria "chemical vaccine", combining the efficacy of a drug (like atovaquone) with the durability of a biological vaccine. Of concern, however, is the possible selection and transmission of drug-resistant parasites. We addressed this question by generating clinically relevant, highly atovaquone-resistant, *Plasmodium falciparum* mutants competent to infect mosquitoes. Isogenic paired strains, that differ only by a single Y268S mutation in cytochrome b, were evaluated in parallel in southeast Asian (*Anopheles stephensi*) or African (*Anopheles gambiae*) mosquitoes, and thence in humanized mice. Fitness costs of the mutation were evident along the lifecycle, in asexual parasite growth in vitro and in a progressive loss of parasites in the mosquito. In numerous independent experiments, microscopic exam of salivary glands from hundreds of mosquitoes failed to detect even one Y268S sporozoite, a defect not rescued by coinfection with wild type parasites. Furthermore, despite uniformly successful transmission of wild type parasites from *An. stephensi* to FRG NOD huHep mice bearing human hepatocytes and erythrocytes, multiple attempts with Y268S-fed mosquitoes failed: there was no evidence of parasites in mouse tissues by microscopy, in vitro culture, or PCR. These studies confirm a severe-to-lethal fitness cost of clinically relevant atovaquone-resistant *P. falciparum* in the mosquito, and they significantly lessen the likelihood of their transmission in the field.

Significance

New tools are needed to protect individuals from malaria and to control malaria in the field. Atovaquone plus proguanil is a commonly used and well-tolerated medicine to prevent malaria. No drug resistance has been reported from its prophylactic use, but tablets must be taken daily. Giving atovaquone as a single injection may provide much longer-lasting protection, against both falciparum and vivax malaria, but there is concern this may create drug resistance. In this study we showed that clinically relevant atovaquone-resistant malaria parasites survive poorly, if at all, in mosquitoes, and that mosquitoes do not transmit drug-resistant parasites to humanized mice. These findings lessen the likelihood that an atovaquone "chemical vaccine" would lead to the spread of atovaquone resistance.

Introduction

Despite concerted efforts, that over several decades successfully cut by half the world's malaria burden, in recent years progress has stalled and appears to be reversing. Estimates in 2021 were of 247 million infections and 619,000 deaths, attesting to malaria's ongoing threat to public health and productivity (1). Of greatest concern are infections with *Plasmodium falciparum*, which comprise 90% of cases and may be lethal, though geographically widespread *P. vivax* also causes significant morbidity. Bednets, vector reduction, various drug regimens, and, most recently, the *P. falciparum* RTS,S vaccine, are means of control, but new strategies are clearly needed. Among the candidates are long-acting injectable antimalarials (2), which for chemoprophylaxis have aptly been termed "chemical vaccines" (3).

Although long-acting injectable drugs are mainstays in contraceptive and antipsychotic therapy, new advances in drug formulation have brought this modality into use against infectious diseases (4-6). Every-two-month injection of viral integrase inhibitor cabotegravir was recently approved as monoprophylaxis against sexually acquired HIV infection (7). Chief among the benefits of long-acting injectable drugs is that they circumvent the commonplace problem of ensuring, over months to years of time, compliance with the frequent dosing required by oral regimens. In previous studies with *P. berghei* ANKA in mice we found that an oral dose of atovaquone protected for one day, but when the same dose was given as a single injection of atovaquone nanoparticles in aqueous suspension, the protection lasted four weeks, a duration expected to be longer in humans (8).

Reflecting the complex lifecycle of malaria parasites within humans (each stage with its distinctive biochemistry and pharmacology), conventional antimalarial drugs have limited scope in human infections. Although it is the sporozoite form that is transmitted by female anopheline mosquitoes, the parasites of critical importance for chemoprophylaxis against symptomatic disease have been the initial (and entirely asymptomatic) liver stage, and the subsequent asexual (pathogenic) erythrocytic stages – targets for causal and suppressive prophylaxis, respectively. Most agents are suppressive (e.g., chloroquine, mefloquine, doxycycline) but the synergistic fixed combination of atovaquone plus proguanil has both causal and suppressive action. Key among the advantages of causal prophylaxis is the substantially smaller number of parasites (difference ≥4 logs) that must be overcome, which reduces the likelihood of failure or resistance.

Atovaquone was devised as a structural mimic of coenzyme Q (or ubiquinone, an essential component in mitochondrial electron transfer), that binds to cytochrome b, collapses the transmitochondrial membrane potential, and impairs the malaria parasite's obligatory synthesis of pyrimidines (9, 10). Atovaquone has garnered a reputation for being safe and well-tolerated when used alone for prophylaxis or treatment of *Pneumocystis jirovecii* or *Toxoplasma gondii* infection, which entails months to years of high dose therapy and plasma levels logs greater than those required for causal malaria prophylaxis (9). Development of atovaquone alone to treat established erythrocytic malaria infections was abruptly paused by the unexpectedly ready appearance of drug resistance, mediated by mutations in *Pfcytb*, the mitochondrial gene encoding cytochrome b, a finding that confirmed the mechanism of action (11, 12). In an effort

to salvage atovaquone, selected from among several possible partners was proguanil, itself also active against liver stage parasites, and itself substantially synergistic with atovaquone. Clear reductions in requisite dose of each partner drug against erythrocytic parasites was shown in laboratory and clinical studies (11, 13). Field studies with atovaquone + proguanil confirmed reliable cures (14), and the causal prophylactic activity of atovaquone alone was demonstrated in humans (15). Accordingly, fixed combination atovaquone + proguanil was FDA approved in 2000 for treatment of malaria. Although the impact of co-administered proguanil on efficacy and resistance against liver stages was not assessed, the same formulation was also approved for causal prophylaxis, making this a notable exception to the usual single drug malaria prophylaxis.

Numerous studies have focused on the issue of atovaquone resistance, pinpointing mutations in loops of cytochrome b that extend into the intermembrane space, where reduced coenzyme Q binds. Mutants have been obtained in vivo (from clinical drug treatment failures or murine models) and from in vitro selection. Curiously, there are marked differences in the mutants obtained clinically versus in vitro. The several dozen literature reports of atovaquone + proguanil treatment failures for *falciparum* malaria all document highly resistant Y268N/S/C mutants (16). (Reported without drug sensitivities are I258M (17) and no mutation in *Pfcytb* (18, 19)). By contrast, in vitro experiments with *P. falciparum* have generated at least 11 different mutations, some multiple, all in the intermembrane space or transmembrane regions of the protein, and with just one exception (20), none at Y268 (12, 20, 21). Furthermore, atovaquone resistance of in vitro-generated mutants is substantially lower (typically 100- to

1000-fold) than that of the clinically relevant Y268N/S/C. The dichotomy between in vitro and clinical mutants assumed greater importance with intriguing reports that the development of atovaquone-resistant parasites in mosquitoes is impaired or halted (22-24), suggesting their transmission may be limited in the field. Unfortunately, these studies have not included an evaluation of the Y268 mutants of *P. falciparum* that pertain to clinical drug resistance.

Transmissibility, or not, of atovaquone resistance is clearly critical to the use of long-acting injectable atovaquone as a chemical vaccine against malaria. In this study we generated a clinically relevant Y268S mutant of *P. falciparum* NF54, and, in comparison to isogenic wild type (WT) control parasites, monitored its growth and differentiation in anopheline mosquitoes and its transmissibility to mice engrafted with human hepatocytes and erythrocytes.

Results

Genesis and in vitro characterization of atovaquone-resistant *P. falciparum*. We were unable to obtain from other sources atovaquone-resistant *P. falciparum* with a Y268 mutation in cytochrome b, that retained the ability to infect mosquitoes. Accordingly, they were generated in vitro by applying atovaquone pressure to mosquito-competent *P. falciparum* NF54 lines, minimally passaged after isolation from clinical trial volunteers.

Over a 21 month period, efforts by several investigators, entailing a total of 7.8 x 10^9 gametocyte-competent parasites, yielded five independent isolates of atovaquone-resistant cells, three of which were Y268S mutants. To select these mutants, continuous drug pressure was applied to various parasite densities and hematocrit levels. Mutants were detected by microscopy 25-47 days after start of pressure (*SI Appendix*, Fig. S1*A,B*). Reasoning that the mutation rate in mitochondrially-encoded *Pfcytb* may be greater in cells cultured in a relatively high oxygen atmosphere (25), selections (and parallel no drug controls) were conducted with cells acclimated to conventional incubator conditions of 5% CO_2 in air (i.e., 19% O_2) (26). This is substantially higher than the 2-3% O_2 in classic cultivation of *P. falciparum* (27), or the ~13% O_2 in our routine candle jar conditions, but it nevertheless falls within the range experienced by parasites in vivo (26). Below-described studies in mosquitoes and mice revealed no difference between parasites maintained continuously in candle jar conditions versus those in 5% CO_2 in air for the ~two-month period of resistance selection.

Parasites that emerged from drug pressure were first evaluated for changes in *Pfcytb*, by PCR amplification and sequencing of the entire open reading frame (*SI Appendix*, Fig. S2*A,B*). Three independent mutants encoded a Y268S amino acid change (*SI Appendix*, Fig. S1*B,C*). Whole genome sequence analysis of mutant versus isogenic wild type parent cells, at a coverage of 26- to 35-fold, revealed no other significant genetic changes, including amplifications or other mutations.

These collective efforts provided a mutation rate of 6 X 10^{-10}. This is somewhat less than the previously reported 4.6 x 10^{-9} for atovaquone against 3D7 (28), and substantially lower than 10^{-5} reported for atovaquone against multi-drug resistant W2 from Indochina (29), perhaps reflecting our use of high drug concentrations for selection, or perhaps there is some impact of the retained (and likely costly to the cell) ability of these parasites to generate infectious gametocytes. Interestingly, all three Y268 mutations were Y-to-S (TAT-to-TCT), as was the only other previously reported in vitro-selected Y268 mutant (20). We did not obtain the Y268N (to-AAT) or Y268C (to-TGT) mutants that are also seen clinically. Although all four known in vitro-selected mutants, from two laboratories, are thus Y268S, and this may have happened by chance, failure to obtain Y268N/C may also reflect a biological bias that operates for selection in vivo but not in vitro.

Asexual erythrocytic Y268S parasites had several phenotypic changes. Their growth rate in vitro was only 71% that of isogenic parent cells (Fig. 1*A*), and they reached lower peak parasitemia, findings consistent with a fitness cost for this mutation (*SI Appendix*, Fig. S3*A*). Compared to

WT, mutant parasites had significantly fewer progeny within late schizonts, a difference that almost certainly contributes to slower growth (*SI Appendix*, Fig. S3*B*). However, the *Pfcytb* mutation and atovaquone resistance remained stable for at least 4 months in the absence of drug pressure, indicating the fitness cost is sustainable for asexual erythrocytic parasites in vitro.

As expected, the Y268S mutation was associated with substantial atovaquone resistance (Fig. 1*B*). Baseline EC_{50} for wild type control parasites was 0.68 nM, in line with published values of less than 1 nM (30), but was 9.0 µM for the Y268S mutant, an impressive change in drug susceptibility also in keeping with previous reports (12, 31). Preliminary experiments in our hands indicated that EC_{50} values for atovaquone can vary 2- to 10-fold depending on protein source in the medium (albumax versus human serum) or differential count of the starting culture. These, in conjunction with atovaquone's limited aqueous solubility at highest assay concentrations, likely contribute to the 75-fold range of EC_{50} values reported by different labs for Y268S clinical isolates (12, 31, 32). Ambient atmosphere (3% O_2, candle jar, or 5% CO_2 in air) had no significant effect on EC_{50} values. Susceptibilities to artemisinin (9.8 versus 12 nM) or chloroquine (8.2 versus 11 nM) were not significantly affected by the Pfcytb Y268S mutation.

The final in vitro assay we conducted was an assessment of male gametocyte exflagellation (Fig. 1*C*). This phenomenon normally occurs in the midgut of anopheline vectors after ingestion of an infected bloodmeal, during which male gametocytes release up to 8 motile gametes (33). This process, induced in vitro by incubating mature gametocytes at room temperature (34),

revealed a highly significant nearly four-fold decrease in exflagellation centers in Y268S mutants as compared to isogenic controls, likely reflecting an increased demand for mitochondrial respiration (35).

Characterization of parasites in anopheline mosquitoes. In all experiments, stage V gametocytemia in infecting bloodmeals was adjusted to be the same, at 0.5%. Ookinetes (both immature and mature) were evaluated in the midguts of blood-fed *Anopheles stephensi* at 20-26 h after infection. The count in mosquitoes infected with WT parasites was nearly 8-fold greater than that in Y268S mutant-fed insects (*SI Appendix*, Fig. S4*A,C,D*).

Oocyst numbers in *An. stephensi*, evaluated in nine independent experiments (Fig. 2*A*), revealed a highly significant difference in the number of wild type or isogenic Y268S mutants (medians 39 or 0 per midgut, respectively). Prevalence of mutants was similarly reduced (Fig. 2*A, pie charts*). Furthermore, although drug-resistant Y268S parasites generated up to 5 oocysts/midgut in 15% of samples, mercurochrome-stained images revealed that their quality was uniformly poor, appearing smaller and malformed relative to their WT parents (Fig. 2*B*,*C*), with median diameter less than half that of WT (*Fig. 2D*).

An. stephensi salivary glands were examined for sporozoites in eight biological replicate experiments (Fig. 2*E*). The difference between WT and Y268S mutant parasite counts was stark: in a total of nearly 250 mosquitoes per group, median counts were 27,700 and zero,

respectively. Difference in the maturation time of mutant versus wild type sporozoites as a possible cause of these discrepant counts was ruled out (*SI Appendix*, Fig. S5).

To test whether the phenotypic differences between wild type and atovaquone-resistant parasites in *An. stephensi* would apply in another major malaria vector, we repeated these experiments with *An. gambiae*, and obtained essentially the same results: ookinetes were less (*SI Appendix*, Fig. S4*B,E,F*), and median counts were reduced from 22 to 0 for oocysts and from 2700 to 0 for sporozoites/mosquito (*SI Appendix*, Fig. S6). As with *An. stephensi*, no sporozoites were seen in any of the Y268S salivary gland preparations.

Since malaria infections in the field are frequently polyclonal, we wanted to determine whether the severe impairment in Y268S sporozoite number could be rescued by coinfection with wild type parasites. *An. stephensi* were fed gametocyte cultures with WT *P. falciparum* only, or with mixtures of 1:1 or 1:10 WT:drug-resistant parasites. In three replicates, median oocyst numbers were 11, 5, and 1 for WT, 1:1 and 1:10 groups, respectively (*SI Appendix*, Fig. S7*A*). Sporozoite counts were similarly patterned (*SI Appendix*, Fig. S7*B*). Thus, although total gametocyte numbers in the feed were constant, the number of tissue parasites, for both oocysts and sporozoites, correlated strongly with proportion of WT gametocytes in the bloodmeal (*SI Appendix*, Fig. S7*C*). In all three replicate experiments, these finding were further confirmed by two complementary RFLP assays, designed to detect 10% or less mutant *Pfcytb* DNA in midgut or salivary gland samples from mixed infections (*SI Appendix*, Fig. S2*D*). All samples evidenced WT DNA, but no mutant DNA was detected in any of the mixed infection

samples (*SI Appendix*, Fig. S7*D*). These findings suggest not only that the cost of the Y268S mutation in mosquitoes is not relieved by coinfection with WT gametes, but indeed that WT parasites may outcompete the mutants in a mixed population.

Transmissibility of *P. falciparum* from mosquito to humanized mouse. To provide a more biologically relevant, and functional, assay for transmissibility of the mutant parasite, huHep mice were bitten by *An. stephensi* infected with either WT or Y268S atovaquone-resistant *P. falciparum* (*SI Appendix*, Fig. S8). In two independent experiments, three mice were bitten by an average of 138 wild type-infected mosquitoes/mouse and two were bitten by an average of 152 mutant-infected mosquitoes/mouse (median parasite intensities in these mosquitoes were comparable to overall data in Fig. 2*A,E*: 29-33 WT or zero Y268S oocysts/midgut; 18,000 WT or zero Y268S sporozoites/mosquito). Engraftment of human erythrocytes, assayed after infusions on days 5.5 and 6 after infection, was robust (WT and mutant averaged 30 and 29%, respectively). By 7.5 d after infection all mice bitten by WT-infected mosquitoes were parasitemic (0.18-0.26%) on Giemsa-stained blood smear (Fig. 3*A*), as confirmed by blood culture. However, no parasites were seen in smears from mice bitten by mutant-infected mosquitoes, and in vitro cultures of 1 mL blood samples harvested at necropsy remained parasite-free for at least three weeks. Confirming these results, blood, liver and spleen from mice infected with WT parasites were positive for both *STEVOR* and *Pfcytb*, but samples from mice exposed to Y268S mutant *P. falciparum* were negative by PCRs (Fig. 3*B,C*).

Discussion

Atovaquone is, in many ways, a uniquely ideal candidate for a long-acting injectable chemical vaccine for malaria. It is potent against both initial liver and pathogenic erythrocytic parasites, for both *P. falciparum* and *P. vivax*; it has a decades-long track record of safety in patients whose plasma drug levels are 100 times those required for causal activity; it is not metabolized and has few drug-drug interactions; and it faces no established resistance in the field or recognized cross-resistance with other antimalarials. No other currently available antimalarial even approaches these characteristics. Exciting candidates in development (2) still require years of nonclinical study and early clinical trials, none of which can provide the extensive human exposure that bears on safety. More practically, further development as a chemical vaccine is facilitated by the fact that atovaquone (alone as Mepron, or in fixed combination with proguanil as Malarone) is off-patent, which, together with extensive use in humans, streamlines its regulatory requirements and facilitates its more equitable deployment.

Countering these advantages is concern about atovaquone resistance. Selected readily when symptomatic patients harboring $10^{9\text{-}12}$ erythrocytic parasites are treated with atovaquone alone, or occasionally in such patients treated with atovaquone + proguanil, clinical drug-resistance is mediated almost exclusively by a single point mutation at the Y268 residue in cytochrome b. In the setting of long-acting atovaquone monoprophylaxis, resistance concerns are twofold. *First* is the possibility that causal therapy may itself select resistant parasites. Several factors mitigate against this, including the fact that drug is present before any parasites are introduced, and that the number of liver stage parasites from which to select a mutant is

relatively small (by orders of magnitude, a recognized "bottle neck" in the lifecycle). This analysis is supported by the absence of reported resistance in people prophylaxed with atovaquone + proguanil (36), despite its being the most widely prescribed agent for this indication (37), and the tens of millions who travel to malarious areas every year (38). *Second* is the concern that mutants occurring in a person dosed with long-acting atovaquone will be transmitted by mosquito to others. Importantly, atovaquone + proguanil is not standard therapy for malaria patients in the field, and there is no cross resistance with other drugs. In the unlikely event of mosquito-borne resistance, the consequence would not be loss of available therapies to treat individual patients but rather loss of atovaquone as a chemical vaccine.

Our studies focused on transmissibility of the clinically relevant highly atovaquone-resistant Pfcytb Y268S mutant *P. falciparum*. In 14 independent experiments, including both *An. stephensi* and *An. gambiae* mosquitoes, by conventional light microscopy and with multiple experienced observers, we found *not one* Y268S sporozoite in salivary glands from 345 mosquitoes. Validating this finding, the functional bioassays with huHep mice were especially important. By multiple criteria, including light microscopy (tail snip blood films), exquisitely sensitive PCR for *P. falciparum* nuclear or mitochondrial genes (liver, blood, spleen), or in vitro culture (of total blood volume), they evidenced no transmission of Y268S parasites. Furthermore, a mixture of mutant and wild type parasites, to mimic polyclonal infections available to mosquitoes in the field, failed to boost sporozoite numbers above those expected for the WT component alone, and instead suggest that wild type parasites out-compete the

mutant. In our hands fitness cost of the clinically relevant Y268S mutation in cytochrome b is severe-to-lethal in the mosquito.

At least three other laboratories have evaluated the survival in mosquitoes, and in some cases transmissibility, of atovaquone-resistant parasites (22-24). None of these reports include a clinically relevant *P. falciparum* Y268 mutant. Previous studies included *P. falciparum* with 5 to 20-fold resistant M133I or V259L, and *P. berghei* with both low and high resistance (M133I, Y268C, Y268N). We also isolated a low-grade L144S mutant, and found that it, like previously reported M133I, generated sporozoites (*SI Appendix*, Figs. S1*B*, S5, S9). The best interpretation for all these studies appears to be that malaria parasites (including *P. falciparum*) with low-level atovaquone resistance can generate microscopically-detectable sporozoites in mosquitoes, whereas those with clinically relevant high-grade resistance cannot. Of obvious interest is the relevance of these various mutants to the use of atovaquone in humans.

The striking difference in EC_{50} values seen for atovaquone-resistant parasites obtained in vitro versus those arising in treated humans is almost certainly related to the disparate drug concentrations that are applied for selection. Atovaquone levels applied in vitro have not exceeded 100 nM, whereas trough plasma concentrations are 5,400 nM (2 µg/mL) in subjects dosed daily for malaria prophylaxis (39), and are even higher in those treated for infection. This in turn suggests that parasites with low-level resistance will not survive in treated humans. In conjunction with our observation that the clinically relevant highly resistant Y268S fares poorly,

if at all, in mosquitoes, these findings mitigate concern about transmissibility of atovaquone-resistance.

The availability of gametocyte-competent wild type *P. falciparum* and isogenic mutants with various degrees of resistance provides a new resource for further studies. For example, with highly resistant Y268S we see fitness costs throughout the lifecycle, starting with a 29% reduced growth rate of asexual erythrocytic stages, and then a progressive loss in the mosquito: 77% reduction in exflagellation, 83% reduction in oocysts, and complete loss of sporozoites (Figs. 1,2). By contrast, sporozoite numbers in our L144S mutant are reduced 63%, but not eliminated (*SI Appendix*, Fig. S9*E*). These findings, in parasites that differ by just a single amino acid residue in Pfcytb, suggest that fitness in the mosquito may be proportional to the degree of atovaquone resistance. On a molecular basis, proportionality between the degree of resistance and fitness implies that mutations in cytochrome b that impair atovaquone binding correspondingly impair catalysis. Highly resistant asexual erythrocytic parasites evidence a mild but detectable growth defect, in keeping with their limited dependence on mitochondrial respiration. However, in the mosquito where mitochondrial function is essential (22), even low-level resistance markedly impacts growth, and high-level resistance is lethal. These findings are akin to previous reports of differences in the pattern of *P. falciparum* drug-resistance polymorphisms in human blood versus mosquito tissues, likely also reflecting dissimilar selective pressures in the hosts, that may impose vector-selective constraints on drug resistance in the field (40).

Our studies indicate that atovaquone resistance may not pose as great a threat to long-acting causal prophylaxis as it did historically to treatment of established erythrocytic infections, and that clinically relevant highly resistant *P. falciparum* mutants are not transmitted by mosquitoes. In view of resurging malaria numbers, limited efficacy and durability of current biological vaccines, and the need for new control strategies, it would seem sensible to establish proof-of-concept for atovaquone as a chemical vaccine. No amount of work can prove the negative (i.e., resistance is not transmissible) and no laboratory investigations can address the enormous diversity of real-world malaria. Despite the promise of our studies, transmissibility, or not, of atovaquone resistance will best be determined by carefully designed and conducted clinical trials in endemic areas.

Materials and Methods

***P. falciparum* cultivation and mutant selection.** Studies were conducted with low passage *P. falciparum* NF54 (MRA-1000 BEI Resources, NIAID, NIH), grown asynchronously at 37°C under candle jar atmosphere (~13% O_2, 3% CO_2, 84% N_2) in RPMI supplemented with 2.1 mM L-glutamine, 25 mM HEPES, 0.25% $NaHCO_3$, and 0.37 mM hypoxanthine, in 10% heat inactivated human serum (Interstate Blood Bank) and O+ erythrocytes at 2 or 4% hematocrit (27). Periodic testing for mycoplasma was negative (MP0025, Sigma-Aldrich). Erythrocytes were obtained weekly from healthy donors under an Institutional Review Board-approved protocol. Prior to atovaquone pressure, parasites were adapted over 14 d to 5% CO_2 in air, as described previously (26).

For mutant selection, atovaquone (PHR1591, Sigma Aldrich) in DMSO (D128500, ThermoFisher) was further diluted in medium containing 10% serum such that final DMSO was ≤0.1%. Cultures were exposed in parallel to atovaquone or no drug (*SI Appendix*, Fig. S1*A, B*). Medium was replaced every other day; cultures were split 1:2 every 7 d; only the first split was retained. Cells were maintained under constant drug pressure for 60 days or until healthy parasites appeared. Upon confirmation of Y268S mutation, atovaquone was removed and drug-resistant lines and their concurrent no drug controls were returned at once to candle jar conditions, amplified, and aliquots were frozen (Glycerolyte 57, McKesson General Medical). In all subsequent experiments WT and mutants were manipulated and studied identically and in parallel.

To obtain gametocytes, cultures were initiated at 0.5% parasitemia, 4% hematocrit, and medium was replaced daily for 15-19 d until gametocytes reached stage V.

***In vitro* drug sensitivities.** Asynchronous *P. falciparum* (0.25% parasitemia, 1.2% hematocrit) in microtiter plates was exposed to serial dilutions of drug (in quadruplicate) for 72h. [^{3}H(G)]-hypoxanthine (16Ci/mmol, 1 mCi/mL NET177001MC, Perkin Elmer) was added for the final 24h, and incorporated radiolabel was evaluated in harvested cells, as described previously (41). EC_{50} values were obtained by nonlinear regression analysis (Prism 6; GraphPad).

***Pfcytb* and whole genome sequences.** For *Pfcytb* sequence of parasites surviving drug pressure, lysates were prepared from cultures at 1% parasitemia (90 °C, 5 min), and a 1848 bp fragment (containing the 1131 bp *Pfcytb* open reading frame) was amplified with primers (*SI Appendix*, Fig. S2*A,B*) and Phusion DNA polymerase (NEB). PCR product was verified by gel electrophoresis as single band of predicted size, and sequenced. Sequences were aligned to *P. falciparum* 3D7 cytochrome *b* (PF3D7_MIT02300) using NCBI blast, and positions on all trace files were assessed in SnapGene.

For whole genome sequences to identify the causative mutation (42), DNA was isolated from 10^8 asexual parasites; QiAamp DNA Mini Blood kit, Qiagen), quantified (Qubit HS ds DNA assay (Qubit Flex Fluorometer, ThermoFisher)), and quality assessed by Genomic Screentape analysis (TapeStation 2200, Agilent). Barcoded libraries for DNA-Seq were synthesized from 100 ng DNA (Celero EZ-Seq kit with NuQuant, Tecan Genomics), quality assessed by High Sensitivity DNA Lab

Chips (BioAnalyzer 2100, Agilent), quantified by Qubit HS dsDNA assay, and sequenced on Illumina's MiSeq platform, (2 x 300bp v3 with 5% PhiX; JHMI Synthesis and Sequencing Facility). Using Partek Flow with sequence defaults, analyses included pre-alignment QA/QC, adaptor/read trimming, reference genome alignment to *P. falciparum* NF54 (PlasmoDB) using Bowtie 2, post-alignment QA/QC, FreeBayes variant calling, variant filtering (VarQual ≥ 30), and variant annotation based on precedence rules. Sequence files for WT parent and Y268S mutant were deposited (NCBI Sequence Read Archive, BioProject ID: PRJNA913198). To identify changes unique to atovaquone-resistant parasites, sequences were compared to that of the isogenic parent line, and then to those of seven other NF54-derived atovaquone-sensitive strains.

Gametocyte exflagellation. Just prior to mosquito feed, 200 μL of gametocyte culture was taken to assess male exflagellation (43, 44). Briefly, cultures were centrifuged in a warm tube (500 x g, 5 min), 2 μL of cell pellet was placed on a glass slide, covered, and incubated (room temperature, 15 min). The number of exflagellation centers in 11 independent 400X fields was counted and normalized to 1.5% gametocytemia. In three of four experiments, counts were obtained in a blinded fashion.

Mosquito maintenance and infection. All experiments were conducted with *An. stephensi* (Liston, NIH) or *An. gambia*e (Keele, NIH) reared and maintained in the Johns Hopkins Malaria Research Institute insectary (27 °C, 80% humidity, 12/12-h light/dark cycle), and provided 10% sucrose solution-soaked cotton pads (43). For each experimental group, 100-200 mosquitoes at

3-7 d post emergence were fasted overnight prior to 37 °C water-jacketed glass membrane feeding, for one hour, by established protocols (43). All bloodmeals were adjusted to 0.5% stage V gametocytemia. Thereafter, 10% sucrose was withheld for 48 h to eliminate unfed mosquitoes.

Ookinete, oocyst and sporozoite counts. Ookinetes were counted by an adaptation of established protocols (45). At 20-26 h post bloodmeal, midguts were dissected from ten visibly blood-fed mosquitoes, placed in 20 µL 3% acetic acid, and vigorously pipetted to lyse midguts. An aliquot (2 µL) was loaded on a hydrophobic slide (VWR, 100488-892), spread to fill a 14 mm circle, dried, fixed with methanol, and stained with Giemsa. For each experimental group, the number of mature and immature ookinetes in sixty 1000X fields was recorded.

Oocyst and sporozoite counts were obtained by adaptation of established protocols (43). At 9-10 d post feed, 15-30 midguts per group were dissected into 1X PBS on a hydrophobic slide (VWR, 100488-892). Samples were stained with 0.1% mercurochrome (M7011, Sigma Aldrich), covered, and examined (100X) for oocysts. At 17-20 d post feed, salivary glands from 20-38 mosquitoes per group were collected, transferred to 100 µL 1X PBS on ice, sedimented (5000 g, 30 sec), and homogenized (sterile pestle, 30 sec). Parasites were counted by hemocytometer to obtain an average sporozoite count per mosquito.

Transmission from *An. stephensi* to huHep mice. All animal studies were conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee with huHep mice

(FRG KO (Fah–/–, Rag2–/–, Il2rg–/–) on NOD background) engrafted with human hepatocytes and capable of maintaining circulating human erythrocytes (Yecuris; (46, 47)). Mice (27-28 wk old) were housed with sterile water, chow, and bedding, and until ≥8 d prior to infection were maintained on 1 mg/mL 2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedione (CuRx™ Nitisinone, 20-0027) in sterile 10% dextrose. Mosquitoes (17-18 d post-infection with WT or isogenic atovaquone-resistant parasites) were fed through a mesh-covered container on mice anesthetized with 4-6 µL/g ip of 14 mg/mL ketamine (67457010810, Myland Pharmaceuticals) plus 1.2 mg/mL xylazine hydrochloride (X1251-1G, Sigma Aldrich) in PBS. Mosquitoes fed for 14 min, with interruption every two min to limit blood loss in mice; over 80% evidenced having taken blood. At 5.5 and 6 d post mosquito probe, mice were injected intravenously with 600 µL human blood (50% red cells, 25% O+ serum, 25% RPMI). Human erythrocyte engraftment was assayed by flow cytometry of peripheral blood incubated with APC-conjugated TER-119 antimouse monoclonal antibody (BD Biosciences), with gates at APC^+ and APC^- (mouse and human erythrocytes, respectively) (Attune NxT Flow Cytometer, BD Biosciences).

Starting 6.5 d after infection, Giemsa-stained blood films were monitored twice daily. At patency, all mice were sacrificed. Blood was collected by cardiac puncture into citrate/phosphate/dextrose (Sigma, C7165), for PCR (150 µL aliquot) and in vitro culture (the ≥1 mL residual blood volume). After perfusion with 15 mL 0.9% NaCl, liver and spleen were harvested, weighed, and stored at -70 °C. Blood cultures were maintained for three weeks or until a robust parasitemia was evident.

PCR assays. Mouse tissues were assayed for *STEVOR* or *Pfcytb* by nested PCR. DNA was isolated from blood (QiaAmp DNA Mini Blood Kit, Qiagen; or Monarch Genomic DNA purification kit, NEB) or from perfused organs homogenized (Fisher Bead Mill 4, 2.4 mm metal beads) prior to extraction (DNA Maxi Kit; Qiagen). For *STEVOR* assay, eluates containing DNA (from 25 µL whole blood, or 2 µg DNA from liver or spleen (Nanodrop 2000, ThermoFisher)) were analyzed as described previously (48, 49). For *Pfcytb* analysis, the 25 µL primary amplification reaction contained 50 nM each primer (*SI Appendix*, Fig. S1*A,C*), 200 µM each dNTP (NEB), 1 U Phusion DNA polymerase (NEB), and 10 ng template DNA. The 25 µL nested reaction contained 200 nM each primer (*SI Appendix*, Fig. S1*A,C*), 200 µM each dNTP, 1 U of Phusion DNA polymerase, and 1 µL of primary reaction product. Thermocycling conditions for both reactions were: denaturation (98°C, 3.5 min); 25 cycles of (denaturation (98°C, 30 sec), annealing (56°C, 40 sec), and extension (72°C, 45 sec)); and final extension (72°C, 5 min). Products were separated by agarose gel electrophoresis and visualized by ethidium fluorescence. Serial dilutions into whole blood of a known number of *P. falciparum*-infected erythrocytes indicated both methods detect ≥10 parasites per 25 µL blood sample.

Statistical Analyses. Dose-response data were analyzed as described previously (41). Unless indicated otherwise, differences between Y268S mutants and WT controls were assessed by Mann-Whitney test or Fisher's exact test, in GraphPad Prism 9.

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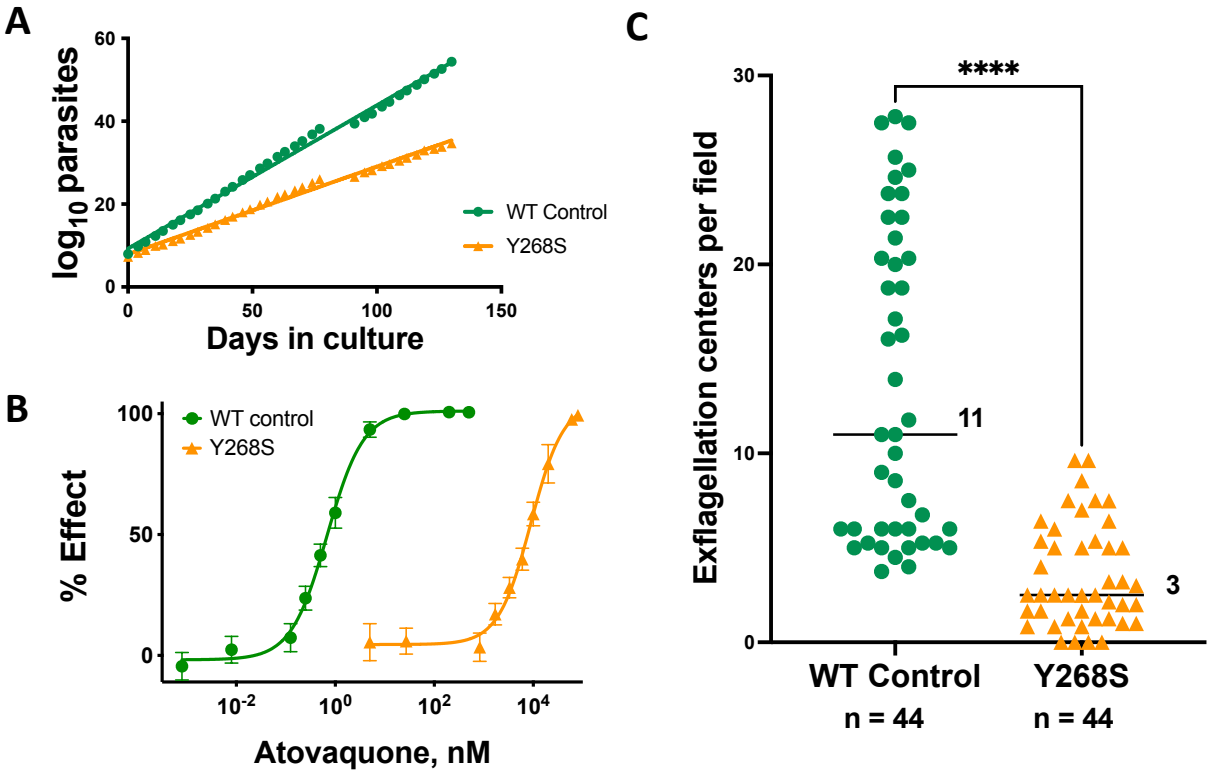

Fig. 1. Phenotypic characterization in vitro of isogenic wild type (WT) and Pfcytb Y268S mutant *P. falciparum*. (A) Cumulative number of asexual parasites in continuous culture. Growth rate of wild type parasites (y = 0.35x + 9.3) was 1.4-fold greater than that of mutant (y = 0.21x + 7.9); *n* = 36, R^2 >0.99. (B) Atovaquone activity against WT or Y268S asexual erythrocytic parasites (EC_{50} 0.68 nM or 9.0 µM, respectively). Depicted are mean ± SD of quadruplicate determinations in three independent experiments (some SD are too small to extend outside the symbols); R^2 ≥0.994. (C) Male gametocyte exflagellation, adjusted to 1.5% gametocytemia. Median number of centers across four independent biological replicates is indicated (ranges 4 to 28 for WT, and 0 to 10 for mutant); *n*, total number of fields examined; ****P <0.0001. For WT cells, all 44

microscopy fields had at least one exflagellation center (100%); Y268S evidenced exflagellation in 40 of 44 fields (91%).

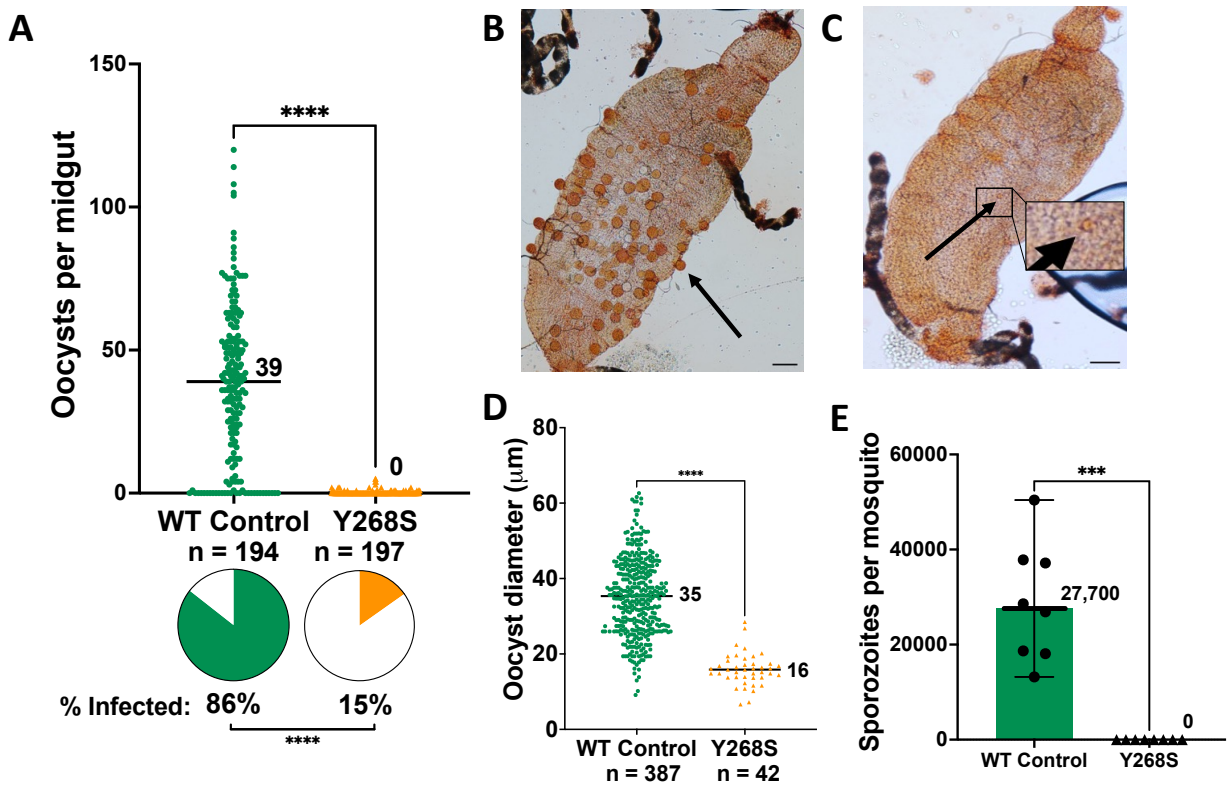

Fig. 2. Evaluation of wild type and Y268S *P. falciparum* in *An. stephensi* tissues. All feeds were adjusted to 0.5% stage V gametocytemia. (A) Oocysts on mosquito midguts were counted at 9-10 d after membrane feed. Indicated is median number of oocysts per midgut in nine biological replicate experiments (ranges 0 to 120 for WT, and 0 to 5 for Y268S); *n*, total number of mosquitoes dissected; *pie charts*, percent of mosquitoes infected; ****P <0.0001. (B, C) Photomicrographs of representative midguts infected with WT or Y268S parasites, respectively. *Bars*, 100 µm; *arrows*, representative oocysts; *inset*, magnification of Y268S oocyst; banded black structures are Malpighian tubules. (D) Oocyst diameters were measured in six biological replicates for wild type, and seven replicates for Y268S parasites. Median diameter is indicated (WT range 9 to 63 µm, mutant range 7 to 28 µm); *n*, total number of oocysts measured; ****P <0.00001. (E) Salivary glands were harvested at 17-20 d after feed in eight biological replicate

experiments entailing a total of 234 WT-fed or 245 Y268S-fed mosquitoes. *Symbols*, for each experiment, average number of sporozoites per mosquito. No sporozoites were seen in any Y268S preparation. *Bars*, median (values indicated) with 95% confidence interval; ***P = 0.0002.

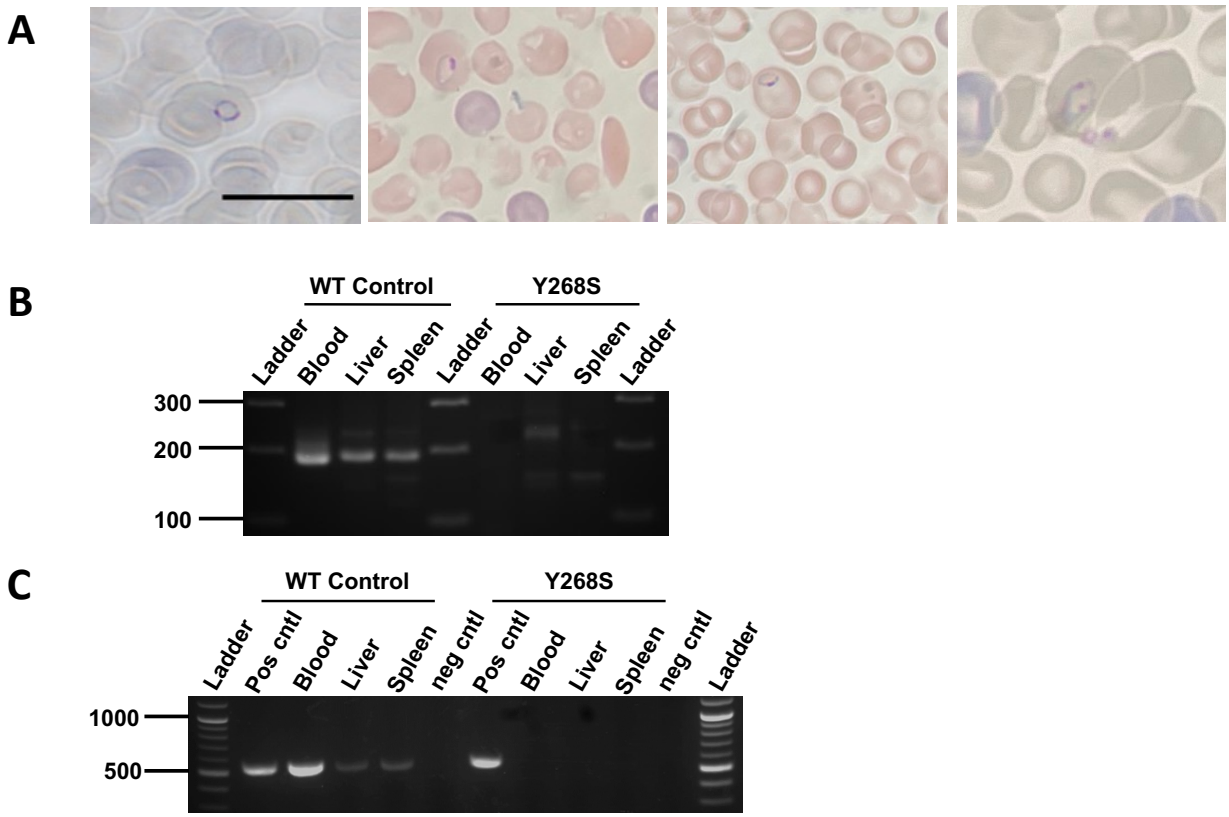

Fig. 3. Analysis of huHep mouse tissues for evidence of WT or Y268S *P. falciparum*. (A) Giemsa-stained thin smears of peripheral blood from mice bitten 7 to 7.5 d previously by *An. stephensi* infected with WT parasites. Intracellular ring stage parasites are evident within the engrafted human erythrocytes, which at 6.4 µm are appreciably larger than the endogenous 4.7 µm mouse cells. Samples are from two mice in each of two biological replicate experiments; *bar*, 10 µm. Analogous peripheral blood from mice bitten by Y268S-infected mosquitoes evidenced no parasites in ≥16,000 RBCs examined per mouse, by at least two independent observers. (B) Indicated tissues were harvested from mice mouse bitten 7.5 d previously by mosquitoes fed on WT or Y268S 0.5% gametocyte culture; liver and spleen were obtained after exsanguination and saline perfusion. DNA was isolated and analyzed by nested PCR for nuclear DNA-encoded

STEVOR sequence (188 bp product). *Ladder*, indicated size fragments. (C) In a second independent experiment, tissues were harvested as above and isolated DNA was analyzed by nested PCR for mitochondrial DNA-encoded *Pfcytb* sequence (538 bp product). *Ladder*, indicated size markers; *pos cntl*, template DNA isolated from cognate asexual parasite culture; *neg cntl*, no template added reaction.