Parasites resistant to the antimalarial atovaquone fail to transmit by mosquitoes

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Atovaquone, a component of the safe and effective antimalarial medication Malaroné, kills both the blood and liver stages of malaria (1). The rollout of cheap generics is thought to have decreased the use of atovaquone, and atovaquone derivatives are in development (1). Atovaquone is prone to resistance (1), and it has been assumed that this resistance will spread (2, 3), as it has for other antimalarials (4, 5). However, the target of atovaquone, cytochrome b (cytB) (6–9), has unique genetics (10–12) and experiences differential selection across the malaria parasite life cycle (23), which prompted us to investigate whether atovaquone resistance can spread via the mosquito vector.

We tested three atovaquone-resistant strains of the rodent malaria parasite Plasmodium berghei, each with different mutations in their mitochondrial DNA-encoded cytB gene (14, 15), for transmissibility from mouse to mosquito and back to mouse (Table 1).

Anopheles stephensi mosquitoes were fed on mice infected with either the parental P.fHanka strain or one of the three atovaquone-resistant mutants, and sexual development of parasites in mosquitoes was assayed (Table 1). All three atovaquone-resistant parasite lines produced wild-type numbers of active male gametes (exflagellation) (Table 1). Parasites carrying the P.fM1331 and P.fNy268C mutations in their cytB gene were able to self-fertilize, generate ookinetes, and successfully produce oocysts, but the oocysts produced had developmental defects (Fig. 1, A and B, and Table 1). Parasites with the P.fNy268N mutation were defective in the ability to self-fertilize and infect the mosquito host (Table 1) due to severely impaired female gamete activation (Fig. 1C). From 17 atovaquone-resistant parasite lines that were able to generate the sporozoite stages in the mosquito salivary glands or was able to infect a naïve mouse (Table 1). We conclude that the rodent malaria atovaquone-resistant cytB mutants tested—which represent a good cross section of the clinical atovaquone-resistant genotypes, including the common Y268 locus (16)—are unable to transmit from mouse to mouse via A. stephensi mosquitoes when self-fertilizing.

To determine whether the presence of wild-type copies of the cytB gene from one parent can complement a mutation in the other, as observed with deletions of electron transport components encoded in the nuclear genome (17), we first crossed P.fNy268C with an atovaquone-sensitive wild-type cytB carrying a mutation (15) in the nucleus-encoded dihydrofolate reductase (dhfr) gene conferring pyrimethamine resistance (P.fHbStf10N) by pooling blood from separate infected mice and then membrane-feeding mosquitoes. Sporozoites were produced, and all 14 naïve mice bitten by these mosquitoes (three trials) developed blood-stage infections. Genotyping of these progeny (passage (Table 1).
zero (P0) showed that outcrossing had occurred because 2 out of 14 mice carried parasites with both the wild-type and pyrimethamine-resistant (S110N) alleles of \textit{Pfdhfr}. However, all 14 mice carried parasites with only wild-type, atovaquone-sensitive \textit{cytB} alleles; outcrossing did not facilitate transmission of atovaquone resistance.

To explore this further, and quantify the level of outcrossing, we fed \textit{A. stephensi} mosquitoes from mice infected with equal numbers of an atovaquone-sensitive line that constitutively expresses green fluorescent protein (GFP) from the \textit{cytB} gene is encoded on maternally inherited mitochondrial DNA (10–12), which implies that known forms of atovaquone resistance must be maternally inherited. We reasoned that the block in mosquito-stage development when atovaquone-resistant parasites attempt to self-fertilize (Table 1) is due to the mutation in the cytochrome \textit{b} protein in the mitochondrion, which is only carried by the female gamete and effectively renders \textit{cytB} mutant females sterile.

To confirm this, we crossed our three \textit{P. berghei} atovaquone-resistant lines with parasites genetically modified to be either male sterile (genotype \textit{Pb}Y268C) or female sterile (genotype \textit{Pbs}Y268C) (19, 20). If atovaquone resistance is indeed linked to mitochondrial inheritance, we expect normal genetic recombination from crosses to male-deficient parasites but no progeny from attempted crosses to a second female-deficient line. After confirming the phenotypes of the tester parasite lines (18–20), we crossed each of them with our three \textit{P. berghei} \textit{cytB} mutants. Crosses to the male-deficient line resulted in recombinant progeny (Table 2), confirming previous results from outcrossing to wild type. Again, though, all progeny from these crosses had wild-type, atovaquone-sensitive \textit{cytB} genotype (Table 2), so they must have acquired their mitochondria from \textit{Pbs}Y268C female gametes (Fig. 2C).

Crossing atovaquone-resistant lines with the female infertile \textit{Pb}nak-4ko (20) resulted in no progeny in 16 of 17 attempts to transmit to a naïve mouse (Table 2), largely confirming our hypothesis that the atovaquone-resistant mutants are effectively female sterile. In a single instance, parasites carrying the Y268C mutation were transmitted but with a markedly reduced efficiency (8 days to patency) (Table 2). Three independent cloned lines of the parasites recovered from this sole transmission event (named \textit{Pb}Y268C P0) were unable to retransmit when either self-fertilized or backcrossed to \textit{Pb}nak-4ko parasites (Tables 1 and 2); passage had not improved their transmissibility. In sum, from 44 separate transmission attempts involving 750 mosquito bites, atovaquone resistance transmission was only observed once, and this mutant was unable to transmit further despite seven attempts. We conclude that the \textit{cytB} mutations in the mitochondrial DNA of atovaquone-resistant rodent malaria parasites render them effectively female sterile and hence largely unable to pass on the resistance gene.

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### Table 1. Atovaquone-resistant mutants in rodent and human malaria parasites fail to produce sporozoites in mosquitoes, and bite-back experiments with mice yielded no resistance transmission.

<table>
<thead>
<tr>
<th>Parasite genotype</th>
<th>Atovaquone IC\textsubscript{50} (nM)</th>
<th>Number of infections</th>
<th>Exflagellations per 10\textsuperscript{5} red blood cells</th>
<th>Ookinete infected mosquitoes (n = no. of mosquitoes)</th>
<th>Midgut infection infected oocytes per mosquito (n = no. of mosquitoes)</th>
<th>Sporozoites per mosquito (n = no. of mosquitoes)</th>
<th>Transmission to naïve mouse</th>
<th>Time to patency (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Pb} wt/wt</td>
<td>9.1 ± 0.4</td>
<td>6</td>
<td>5.3 ± 1.6</td>
<td>23276 ± 810.7 (58)</td>
<td>100</td>
<td>109.8 ± 21.7 (85)</td>
<td>10,348</td>
<td>3/3</td>
</tr>
<tr>
<td>\textit{Pb} wt/M133I</td>
<td>250 ± 41</td>
<td>4</td>
<td>5.5 ± 1.9</td>
<td>1488 ± 549 (38)</td>
<td>59</td>
<td>32.2 ± 9.8 (69)</td>
<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>\textit{Pb} wt/Y268C</td>
<td>23,695 ± 915</td>
<td>3</td>
<td>4.5 ± 1.3</td>
<td>725 ± 52.0 (30)</td>
<td>50</td>
<td>70 ± 2.9 (47)</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>\textit{Pb} wt/Y268C P0</td>
<td>19,080 ± 1119</td>
<td>4</td>
<td>6.7 ± 1.4</td>
<td>347 ± 970 (29)</td>
<td>52</td>
<td>17.1 ± 10.2 (85)</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>\textit{Pb} wt/Y268N</td>
<td>11,625 ± 1225</td>
<td>6</td>
<td>7.3 ± 1.9</td>
<td>417 ± 41.7* (81)</td>
<td>17</td>
<td>2.7 ± 1.7 (80)</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>\textit{PnF}54e/wt</td>
<td>2.25 ± 113</td>
<td>4</td>
<td>20.0 ± 9.3</td>
<td>1918 ± 225 (30)</td>
<td>973</td>
<td>98.5 ± 38.5 (83)</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>\textit{Pn}F54e/M133I</td>
<td>16.2 ± 3.9</td>
<td>4</td>
<td>9.3 ± 3.8</td>
<td>0</td>
<td>1.5 ± 1</td>
<td>0.02 ± 0.02* (144)</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>\textit{Pn}F54e/Y259L</td>
<td>35.3 ± 2.5</td>
<td>4</td>
<td>7.0 ± 4.3</td>
<td>0</td>
<td>3 ± 1</td>
<td>0.03 ± 0.01 (154)</td>
<td>nd</td>
<td>na</td>
</tr>
</tbody>
</table>

*Parasites were detected from only a single experiment.
which must be inherited through the mitochondrion (Fig. 2).

To determine whether the impact of cytB mutations conferring atovaquone resistance on transmission is similar in the human malaria parasite (*P. falciparum*), we selected atovaquone-resistant lines by repeated exposure to sublethal concentrations of a drug during in vitro culture (16). Two clones, with different mutations in cytB (M133I and V299L), were established (Table 1). In vitro cultured gametocytes were fed to *A. gambiae* mosquitoes, and oocyst numbers were counted 7 days after infection (Table 1). The parental line (NF54e) retained normal mosquito infectivity (Table 1). However, the two atovaquone-resistant mutants were severely impaired in their mosquito infectivity and in the number of oocysts produced when infection did occur (Table 1). The severe defect in activation of female gametes phenocopies the reduced number of activated females in the rodent malaria *PbY268N* (Fig. 1C). We conclude that human *P. falciparum* malaria parasites carrying atovaquone resistance mutations in cytB are unable to successfully infect

<table>
<thead>
<tr>
<th>Cross nuclear genotype/ mitochondrial genotype</th>
<th>Number of infections</th>
<th>Exflagellations per 10⁴ red blood cells</th>
<th>% infected oocysts per mosquito (n = no. of mosquitoes)</th>
<th>Sporozoites per mosquito (n = no. of mosquitoes)</th>
<th>Transmission to naïve mice</th>
<th>Time to patency (days)</th>
<th>Nuclear genotype PO</th>
<th>Mitochondrial genotype PO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP/wt x GFP/wt</td>
<td>3</td>
<td>6.07 ± 1.88</td>
<td>93 ± 6</td>
<td>12,433 ± 1822</td>
<td>4/4</td>
<td>4 ± 0</td>
<td>100% GFP</td>
<td>wt</td>
</tr>
<tr>
<td>wt/M133I x GFP/wt</td>
<td>3</td>
<td>6.77 ± 2.16</td>
<td>91 ± 6</td>
<td>10,600 ± 3139</td>
<td>4/4</td>
<td>4.25 ± 0.3</td>
<td>60 ± 4% GFP</td>
<td>wt</td>
</tr>
<tr>
<td>wt/Y268C x GFP/wt</td>
<td>3</td>
<td>1.83 ± 0.73</td>
<td>69 ± 6</td>
<td>3133 ± 2533</td>
<td>3/4</td>
<td>4 ± 0</td>
<td>50 ± 8% GFP</td>
<td>wt</td>
</tr>
<tr>
<td>wt/Y268N x GFP/wt</td>
<td>3</td>
<td>6.73 ± 2.13</td>
<td>77 ± 12</td>
<td>6076 ± 2899</td>
<td>4/4</td>
<td>4.5 ± 0.3</td>
<td>72 ± 17% GFP</td>
<td>wt</td>
</tr>
<tr>
<td>s48</td>
<td>45ko/wt x s48</td>
<td>45ko/wt</td>
<td>1</td>
<td>1.7</td>
<td>60 ± 4% GFP</td>
<td>0/1</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>nek-4ko/wt x nek-4ko/wt</td>
<td>2</td>
<td>9.0 ± 6.2</td>
<td>44 ± 15</td>
<td>4575 ± 4425</td>
<td>2/2</td>
<td>4 ± 0</td>
<td>57 ± 5% GFP</td>
<td>wt</td>
</tr>
<tr>
<td>s48</td>
<td>45ko/wt x nek-4ko/wt</td>
<td>1</td>
<td>10.2</td>
<td>85 ± 15</td>
<td>4575 ± 4425</td>
<td>2/2</td>
<td>4 ± 0</td>
<td>57 ± 5% GFP</td>
</tr>
<tr>
<td>GFP/wt x nek-4ko/wt</td>
<td>2</td>
<td>11 ± 3.8</td>
<td>31.8 ± 26.8</td>
<td>85 ± 15</td>
<td>4575 ± 4425</td>
<td>2/2</td>
<td>4 ± 0</td>
<td>57 ± 5% GFP</td>
</tr>
<tr>
<td>wt/M133I x s48</td>
<td>45ko/wt</td>
<td>1</td>
<td>2.2</td>
<td>67 ± 7.3</td>
<td>18,900 ± 10</td>
<td>1/1</td>
<td>4</td>
<td>wt and s48</td>
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<tr>
<td>wt/Y268C x s48</td>
<td>45ko/wt</td>
<td>1</td>
<td>6.4</td>
<td>100 ± 16</td>
<td>8500 ± 10</td>
<td>1/1</td>
<td>4</td>
<td>wt and s48</td>
</tr>
<tr>
<td>wt/Y268N x s48</td>
<td>45ko/wt</td>
<td>1</td>
<td>8.1</td>
<td>90 ± 8.9</td>
<td>2125 ± 15</td>
<td>1/1</td>
<td>4</td>
<td>wt and s48</td>
</tr>
<tr>
<td>wt/M133I x nek-4ko/wt</td>
<td>4</td>
<td>5.5 ± 1.5</td>
<td>48 ± 14</td>
<td>0 (80)</td>
<td>0/5</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>wt/Y268C x nek-4ko/wt</td>
<td>5</td>
<td>7.3 ± 4.3</td>
<td>24 ± 15</td>
<td>500* (108)</td>
<td>1/7</td>
<td>8</td>
<td>wt</td>
<td>Y268C</td>
</tr>
<tr>
<td>wt/Y268CPO x nek-4ko/wt</td>
<td>3</td>
<td>9.3 ± 4.5</td>
<td>41 ± 10</td>
<td>0 (76)</td>
<td>0/5</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>wt/Y268N x nek-4ko/wt</td>
<td>3</td>
<td>12.6 ± 2.1</td>
<td>43 ± 21</td>
<td>0 (31)</td>
<td>0/3</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

*Sporozoites detected in only one infection trial.*
A. gambiae mosquitoes, which strongly suggest that human malaria parasites will also be unable to transmit atovaquone-resistant mutations efficiently.

Our findings show that common, clinically relevant atovaquone resistance mutations block transmission of malaria by the mosquito vector and that this phenotype is a consequence of maternal inheritance of the mitochondrion. Why are cytB mutations "genetic time bombs" that affect the mosquito stages of the parasite so severely? All clinically recovered atovaquone resistance mutations are in the quinol oxidase (Qo) site of the mitochondrion-encoded cytochrome b protein and prevent atovaquone from displacing ubiquinone from complex III in the mitochondrial electron transport chain (1, 6–9, 16). Importantly, ubiquinone → cytochrome b electron transport operates at only...
minimal levels during the malaria parasite blood phase, which relies solely on aerobic glycolysis (21). Nevertheless, nominal transport is essential, primarily as an electron sink for pyrimidine biosynthesis (22). We hypothesize that the modest levels of mitochondrial electron transport during blood phase offer relaxed selection on cytB—which is multicytoplasm and easily mutable (3, 23)—allowing respiration-deficient mutants (8, 24) with reduced atovaquone binding (9) to be readily selected by drug pressure. However, when these mutants switch to the mosquito phase—which relies on full aerobic respiration with an active tricarboxylic acid cycle (25), robust electron transport (17, 26, 27), and mitochondrial adenosine triphosphatase activity (28)—the respiration deficits of the cytB mutants (8, 24) prevent them from completing their development and generating infectious sporozoites. This results in a block of transmission of atovaquone resistance genotypes to new hosts—a block that cannot be overcome by outcrossing because cytB is maternally inherited.

Cytochrome b is thus a rather unique malaria drug target. Its genetics are constrained by maternal inheritance (10–12, 29), there is no recombination of mitochondrial DNA (23), and markedly different selection regimes in the mammalian versus the mosquito hosts (17, 25, 26, 28) all combine to restrict the parasite’s options to disseminate mutations conferring resistance to atovaquone, even though they can arise relatively quickly in patients (2, 9). These constraints likely apply to other cytchrome b targeting drugs currently under development (30–32) and perhaps to drugs targeting the maternally inherited apicoplast (10–12, 29), an endosymbiotic organelle drug target that also has differential activity across the life cycle.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1
References (33–39)
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CELL BIOLOGY

Nuclear envelope rupture and repair during cancer cell migration

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During cancer metastasis, tumor cells penetrate tissues through tight interstitial spaces, which requires extensive deformation of the cell and its nucleus. Here, we investigated mammalian tumor cell migration in confining microenvironments in vitro and in vivo. Nuclear deformation caused localized loss of nuclear envelope (NE) integrity, which led to the uncontrolled exchange of nucleo-cytoplasmic content, herniation of chromatins across the NE, and DNA damage. The incidence of NE rupture increased with cell confinement and with depletion of nuclear lamin, NE proteins that structurally support the nucleus. Cells restored NE integrity using components of the endosomal sorting complexes required for transport III (ESCRT III) machinery. Our findings indicate that cell migration incurs substantial physical stress on the NE and its content and requires efficient NE and DNA damage repair for cell survival.

The nuclear envelope (NE), comprising the inner and outer nuclear membranes, nu- CLEAR, and the nuclear lamina, presents a physical barrier between the nuclear interior and the cytoplasm that protects the genome from cytoplasmic components and establishes a separate compartment for DNA and RNA synthesis and processing (1). Loss of NE integrity and nuclear pore selectivity has been linked to the normal aging process and a variety of human diseases, including cancer (2). In cancer progression, key steps of tumor cell invasion depend upon deformation of the nucleus into available spaces within the three-dimensional tissue (3–6). Whereas the cytoplasm of migrating cells can penetrate even submicron- sized pores, the deformation of the large and relatively rigid nucleus becomes a rate-limiting factor in migration through pores <25 μm2 in cross section (4, 6–10). We hypothesized that migration through such tight spaces provides a substantial mechanical challenge to the integrity of the nucleus. Thus, we investigated whether cell migration through confining spaces induces NE rupture and compromises DNA integrity.