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Supplementary Materials for

Parasites resistant to the antimalarial atovaquone fail to transmit by mosquitoes

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Materials and Methods

Rodent malaria transmission

Atovaquone resistant mutants are as previously described(*14, 15*). All animal experiments were in accordance with the Australian Prevention of Cruelty to Animals Act 1986 and the Prevention of Cruelty to Animals Regulations 2008 and reviewed and permitted by the Melbourne University Animal Ethics Committee (Ethics ID: 0810992.4 and 1112043.1) and Jichi Medical School (Ethics ID: 18-205). Four-week-old male Swiss Webster mice (Monash Animal Research Platform, Melbourne, Australia) were infected by intravenous injection of $1x10^5$ infected red blood cells. 1 µl of tail prick blood was mixed with 100 µl of exflagellation media (RPMI [Invitrogen] supplemented with 10% foetal bovine serum, pH 8.4), incubated for 15 min, and exflagellation events per $1x10^4$ red blood cells were counted. *An. stephensi* (MR4) mosquitoes were allowed to feed on anaesthetised mice once the exflagellation rate was between 2-10 events per 10^4 red blood cells. Twenty-two hours after biting, mosquito midguts were dissected and the blood meal isolated, stained with Giemsa, and ookinetes were counted. Twelve days after biting, mosquito midguts were dissected and visually checked for the presence of oocysts. At least ten mosquito midgut contents were examined from 3-6 separate feeding experiments for each line. Twenty-two days after biting, salivary glands were dissected and checked for the presence of sporozoites. Finally, these mosquitoes were used in bite back experiments with naïve mice. Transmission (number of days till blood stage malaria patency was observed) was checked by daily tail prick and blood smearing for 12 days after biting. Blood samples were taken from mice and genomic DNA was extracted using the NucleoSpin blood kit (Machery & Nagel) for PCR amplification (3700a – TGGATGGTGCTTTAGATATATGC, 4615 – GTTTGCTTGGGAGCTGTAATC) and sequence genotyping (4086 – CCTTTAGGGTATGATACAGC, 4103 – TGATGTATCATACCCTAAAG) of *cytB*.

Oocyst microscopy

Dissected mosquito midguts were fixed in 2.5% glutaraldehyde in PBS, and then in 0.5%OsO_4, dehydrated in an ethanol series, embedded in London Resin White, and semi-thin sections (400nm) mounted on glass slides and stained with 0.5% toluidine blue (w/v): 0.1% Na_2CO_3 (w/v) for 10 seconds, and then imaged on an Olympus BH-2 light microscope.

Gametocyte activation assays

Mosquitoes were fed with atovaquone resistant and parental lines and midguts were dissected at 20 hours post infection and homogenized in PBS, and then a fraction of the homogenate was smeared on a slide and stained with either anti-*Pbs*21 antibody(*33*) for rodent malaria parasites or anti-*Pfs*25 antibody(*34*) for human malaria parasites to identify activated gametocytes expressing the activated gametocyte protein on their surface. The presence of unactivated female gametocytes was confirmed by RT-PCR as described(*35*).

Rodent malaria crosses

Donor mice were pre-infected with frozen stocks, and after 3 to 5 days, $2.5x10^5$ parasites of each line being crossed were injected intravenously into recipient mice. Blood samples were later taken from recipient mice for PCR confirmation of the appropriate dual infections. Mosquitoes were infected as above, and oocyst counts, sporozoite counts, transmission to naïve mice (passage zero/P0), and progeny genotyping were done as above.

Drug selection of human malaria parasites

Gelatine selected *P. falciparum* (NF54e strain, Walter Reed National Military Medical Center, Bethesda) was grown *in vitro* (*36*) and exposed to increasing concentrations of atovaquone(*16*), beginning at 1nM and increasing twofold each time resistant parasites were observed. The parental line experienced the same number of passages without drug. Gelatine selection was repeated before each increase in drug concentrations. *cytB* genes were amplified by PCR (379SP – CTCTATTAATTTAGTTAAAGCACAC, 380ASP – ACAGAATAATCTCTAGCACC) and sequenced (381 – AGCAGTAATTTGGATATGTGGAGG, 382 – AA\TTTTTAATGCTGTATCATACCCT) from two lines able to grow at 8nM atovaquone to characterize possible resistance mutations. Drug resistance was measured *in vitro* as described(*37, 38*).

Transmission of human malaria to mosquitoes

In vitro gametocyte cultures (*Pf*NF54e, *Pf*M133I and *Pf*V259L) were diluted to 0.1% and 0.3% gametocytaemia and fed to *An. gambiae* (Keele) mosquitoes(*39*) on artificial membrane feeders. Fed mosquitoes were kept at 25 °C in a humidified chamber. Mosquito midguts were dissected seven days after feeding and oocyst number per mosquito was determined after staining with 0.2% Mercurochrome.

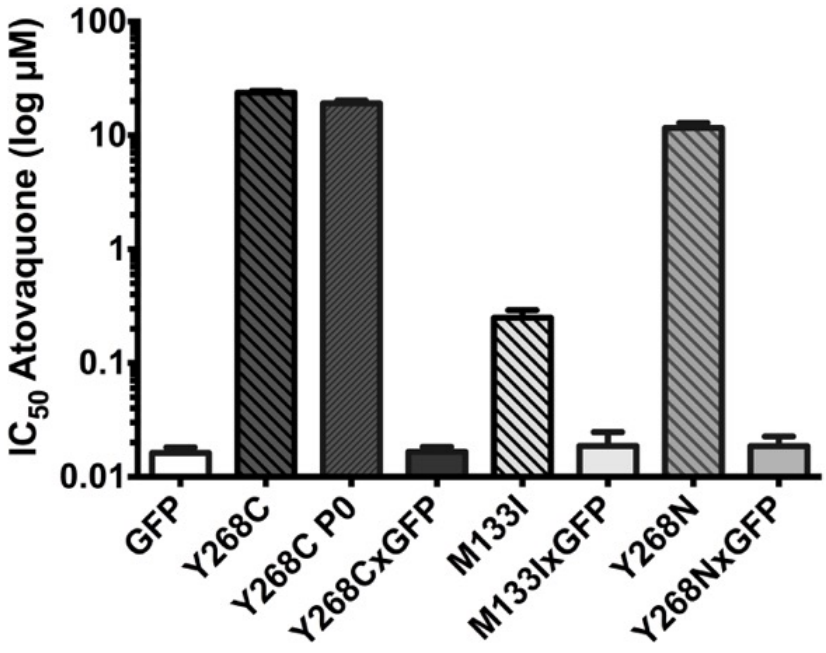

Fig. S1.

Progeny of outcrossing remain sensitive to atovaquone. Atovaquone sensitivity in *ex vivo* assay of parental strains and the progeny of genetic crosses. Bars represent IC_{50} concentrations with SEM. Progeny of genetic crosses are not significantly different to each other or GFP control (one-way ANOVA, $p>0.05$)..

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