Altered drug susceptibility during host adaptation of a *Plasmodium falciparum* strain in a non-human primate model

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Infections with *Plasmodium falciparum*, the most pathogenic of the *Plasmodium* species affecting man, have been reduced in part due to artemisinin-based combination therapies. However, artemisinin resistant parasites have recently emerged in South-East Asia. Novel intervention strategies are therefore urgently needed to maintain the current momentum for control and elimination of this disease. In the present study we characterize the phenotypic and genetic properties of the multi drug resistant (MDR) *P. falciparum* Thai C2A parasite strain in the non-human *Aotus* primate model, and across multiple passages. *Aotus* infections with C2A failed to clear upon oral artesunate and mefloquine treatment alone or in combination, and *ex vivo* drug assays demonstrated reduction in drug susceptibility profiles in later *Aotus* passages. Further analysis revealed mutations in the *pfcr* and *pfdhfr* loci and increased parasite multiplication rate (PMR) across passages, despite elevated *pfmdr1* copy number. Altogether our experiments suggest alterations in parasite population structure and increased fitness during *Aotus* adaptation. We also present data of early treatment failures with an oral artemisinin combination therapy in a pre-artemisinin resistant *P. falciparum* Thai isolate in this animal model.

Antimalarial drug resistance is one of the greatest threats to the current malaria eradication agenda1. Oral artemisinin-based combination therapy (ACT) is the standard of care for uncomplicated malaria, while parenteral intravenous (i.v.) treatment is used in severe cases. Artemisinin (QHS) is the fast acting component of ACTs that accelerates clearing of young ring stage parasites by the spleen2. In 2009 decreased susceptibility to AS was first observed in western Cambodia3, and has since been detected in the rest of Southeast Asia, including recent reports of clinical treatment failure of ACTs such as artesunate/mefloquine (AS/MQ) or artemether/lumefantrine (AL or Coartem)4,5. *In vitro* selection experiments followed by genome-wide association studies have meanwhile identified mutations in the PF3D7_1343700 (*PF13_0238*) genomic locus encoding a Kelch protein that are strongly correlated with slow clearing parasites6. Such mutations appear to occur in the context of a specific genetic background in Southeast Asia but so far have not been detected in Africa7,8.

The TM90C2A (C2A) parasite is a multidrug resistant *P. falciparum* strain originally isolated from a patient in Thailand in 1992, prior to the observation of altered susceptibility to QHS in Southeast Asia9. Initial genotyping

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demonstrated presence of quadruple-mutations in the \textit{dhfr-thymidylate synthase} gene\(^{10}\) and increased \textit{pfmdr1} copy number\(^{11}\). This combination of mutations and \textit{pfmdr1} copy number variation (CNV) has been associated in field isolates with higher \textit{in vitro} inhibitory concentrations to MQ, quinine (QN), halofantrine and QHS, and with failure of MQ monotherapy and AS/MQ combination therapy at the Thai-Cambodian border\(^{22,23}\). Notably, AS/MQ was deployed in 1994 in Thailand and adopted as standard treatment by the Thai health authorities in 2005. However, QHS monotherapy had been used in western Cambodia since the late 1970’s and MQ was introduced in Thailand in 1984\(^{3}\).

Recently, we reported the adaptation of the Thai C2A clone to splenectomized \textit{Aotus l. lemurinus} monkeys (Panamanian Owl monkey). \textit{Plasmodium} infection in \textit{Aotus} was first reported in Panama at the Gorgas Memorial Laboratory in 1966 and has since been established as a major non-human primate model to host-parasite-vector interactions, immunology and pathophysiology and evaluation of drugs and vaccines in \textit{P. falciparum} and \textit{P. vivax}\(^{14}\). During the adaptation process of passaging the parasite in \textit{Aotus}, we observed decreasing susceptibility of the parasites to oral MQ or i.v. AS alone or in combination\(^{15}\). To determine the basis for the altered drug sensitivity phenotype during \textit{Aotus} adaptation, we tested the antimalarial drug efficacy to MQ and artemisinin derivatives \textit{in vivo} and \textit{in vitro} in C2A, and correlated these parameters with growth rates, genotypic changes and mutations in antimalarial drug resistance genes.

Results

**Artesunate treatment failure with the C2A \textit{P. falciparum} strain in the \textit{Aotus} model.** We previously demonstrated treatment failures upon AS treatment with the multidrug resistant C2A in the \textit{Aotus} monkey model\(^{15}\). Specifically, we observed recrudescence in monkey passage VII and suppression in passage VIII, in each case after treatment with AS at 20 mg/Kg i.v. for three days\(^{15}\). To systematically investigate this drug susceptibility phenotype we performed a controlled experiment in \textit{Aotus} and followed parasitemia upon oral AS treatment alone, or in combination with MQ. Malaria naive and splenectomized male and female \textit{Aotus lemurinus lemurinus} monkeys were inoculated with 5 × 10\(^{6}\) parasites of the \textit{C2A} clone on its serial passage from a donor \textit{Aotus} and further divided into three oral treatment groups of two monkeys each (Fig. 1, Table 1); one group was treated with a single dose of 40 mg/Kg of oral MQ; a second group was treated with daily doses of 33 mg/Kg of oral AS for three days; and a third group was treated with a combination of a single dose of oral MQ and three daily doses of oral AS. As a control a spleen intact animal was also infected and treated with the same dose regimen as the third group. Parasitemia was patent in all animals between days 1–2 post infection (PI), reaching peak parasitemia of > 100,000 parasites/µL in groups 1–3 by day 9–11 PI. In contrast, a peak parasitemia of 340 parasites/µL on day 16 PI was reached in the spleen intact animal. On the first day > 100,000 parasites/µL were reached treatment was initiated (Day 0 in Fig. 1).

In each of the three treatment regimes, the animals remained parasitemic with 10–100 parasites/µL for > 10 days (Fig. 1A, Table 1 and S3). In the spleen intact animal, parasitemia persisted at low levels until treatment on day 16 PI and was cleared by day 3 of treatment (Fig. 1B). In contrast, when splenectomized animals were infected with parasites from \textit{Aotus} passages III and IV and treated i.v. with AS at 33 mg/Kg for 3 days and MQ at 40 mg/Kg once at peak parasitemia (< 100,000 parasites/µL), parasitemia were cleared on average on day 5 following initiation of treatment (Fig. 1C, Table S3). Similar clearance times are found in human cohorts: in one study 25% of patients with parasitemia > 4% (> 100,000 parasites/µL) were positive beyond day 3 of treatment, but only 5% with parasitemia < 4%\(^{16}\). Notably, the i.v. AS dosage used in our study is about eight-fold higher than what is commonly given to humans for oral treatment of uncomplicated\(^{17,18}\) or severe malaria (33 mg/Kg versus 4 mg/Kg)\(^{19}\). Converting the \textit{Aotus} animal dose of AS used in this study (33 mg/Kg) to a human equivalent dose based on body surface area\(^{20}\) would result in ~11 mg/Kg, approximately three times the human standard dose of 4 mg/Kg. Together these experiments demonstrate that infections with the MDR C2A isolate in the \textit{Aotus} \textit{P. falciparum} non-human primate model show characteristics of MQ resistance and reduced AS susceptibility, including parasite suppression upon i.v. or oral AS at 33 mg/Kg × three days, or in combination with MQ at 40 mg/Kg once (Fig. 1).

**C2A shows altered drug susceptibility profiles \textit{ex vivo}.** To further investigate the drug resistance profile of the C2A strain we performed \textit{ex vivo} assays of C2A from several \textit{Aotus} passages with a series of antimalarial compounds. Specifically, we determined the \textit{ex vivo} IC\(_{50}\) values for \textit{Aotus} passages II and X and the original \textit{in vitro}-adapted TM90C2A isolate against MQ, CQ, atovaquone (ATV), QHA, DHA and AS (Fig. 2). As a control we also measured the IC\(_{50}\) of the sensitive D6 strain (CQ and MQ susceptible). These experiments confirmed that TM90C2A is indeed resistant to MQ, CQ and ATV (Fig. 2A), as observed in our previous experiments in the \textit{Aotus} model\(^{15}\). MQ IC\(_{50}\) are > 30 nM, a concentration that has been associated with treatment failures in infected patients\(^{21,22}\). Interestingly we also observed reduced susceptibility with C2A to the three different artemisinin derivatives, QHA, DHA and AS, and compared to D6 (Fig. 2B). IC\(_{50}\) concentrations were above the previously defined thresholds of 12 nM\(^{22}\) for DHA (C2A passage X), and 20 nM\(^{21,22}\) for AS (C2A passages II and X). Interestingly, parasites showed a significant decrease in susceptibility against MQ, QHS and DHA between \textit{in vitro}-adapted TM90C2A and C2A passages II and X. In contrast, CQ IC\(_{50}\) was not significantly different across C2A passages. To further evaluate possible reduction in artemisinin susceptibility across C2A passages, we performed the recently established ring stage assay (RSA)\(^{15}\). This experiment indeed demonstrated some reduced susceptibility for \textit{ex vivo} C2A passages II and X compared to the \textit{in vitro}-adapted original TM90C2A isolate and the D6 control (Fig. 2C), although still below the defined threshold of 1% ring stage survival for resistance\(^{21}\). Altogether these data demonstrated phenotypic changes in C2A across \textit{Aotus} passages such as altered susceptibility to QHS, DHA, and MQ. Interestingly the \textit{in vitro}-adapted TM90C2A did not show any signs of altered artemisinin susceptibility while being equally resistant to MQ and CQ compared to the \textit{Aotus} passages.
Genetic analysis of C2A during Aotus passages and in vitro adaptation. To track possible changes in the genetic composition of C2A across Aotus passages and during in vitro adaptation we first analyzed copy number variation (CNV) of *pfmdr1* (Fig. 2D). These experiments demonstrated variation in *pfmdr1* copy number between 2 and 2.5 across all passages, and copy number of 2 in the in vitro-adapted line, suggesting presence of mixed genotypes across Aotus passages.

Given the *pfmdr1* CNV data and the observed drug resistant profiles with C2A, we performed detailed genetic analysis of C2A across passages and with the in vitro-adapted TM90C2A. To determine whether C2A represented...
Figure 2. *Ex vivo/in vitro* antimalarial drug susceptibility and Pfmdr1 copy number variation of PfC2A across Aotus passages. (A,B) IC_{50} antimalarial drug nM concentration bar plots of reference strains D6 (white bar), *in vitro* culture adapted TM90C2A (blue bar) and pfC2A Aotus adapted passage levels II (black bar) and X (red bar). Drug IC50 nM resistance threshold (black line). Mean ± sample SEM (see also Table S1). (C) DHA drug susceptibility based on RSA. Survival percentage = DHA treated parasites/DMSO treated parasites × 100. Red bar represents survival of artemisinin resistant positive control strain KH001-029. D6 is included as negative reference strain. Significant survival threshold (black line) is set at 1% as in Witkowski et al.24. (D) Passage level *pfmdr1* copy number fold change of a *Plasmodium falciparum* C2A clone during adaption to Aotus monkeys. *P. falciparum* strains with two *pfmdr1* gene copies are considered resistant to MQ. Mean ± sample SEM. Dashed line indicates threshold for *pfmdr1* CN level indicative of MQ resistance. Dotted line indicates threshold for *pfmdr1* CN level indicative of MQ sensitivity. MQ = Mefloquine; CQ = Chloroquine; ATV = Atovaquone; AS = Artesunate; QHS = Artemisinin; DHA = Dihydroartemisinin. p = Mann-Whitney U significance t test unpaired samples. ***p < 0.005; **p < 0.05.
a single genotype across passages, despite the observed \( pfmdr1 \) CNV, we performed genotyping using a molecular barcode assay that assesses the parasite genotype based on a combination of 24 neutral loci across the 14 \( P. falciparum \) chromosomes\(^25\). Genotyping by molecular barcode assay demonstrated that C2A parasites across all passages from TM90C2A to passage X show identical or very closely related major haplotypes (Fig. 3A). Genetic analysis of drug resistance loci revealed resistance mutations across all passages including a mutation in the \( PfCFR \) within the \( pfcr \) (M74I, N75E, N76T, A220S, N326S, I356T) and \( PfDHFR \) (N51I, C59R, I164L, S108N) loci (Fig. 3B). No mutations were found in the \( Kelch K13 \) locus across TM90C2A and passage II, III and X. However, compared to the 3D7 reference sequence we found a tandem asparagine insert (NN) after codon 142 in the propeller domain in passages II, III and X as well as in the \( PfCFR \)-adapted TM90C2A. This mutation has been observed in other parasites and is not related to an artemisinin resistance phenotype\(^26\). Interestingly specific mutations in \( pfmdr1 \) (Y184F), \( PfDHPS \) (A437G, A581G) and \( PfATPase \) (L263A) were only found in passages II and III (Fig. 3B). Similarly distinct sets of mutations were found in the \( PfCFR \)-adapted TM90C2A in \( PfDHFR \) (N51Y, C59M) and \( PfATPase \) (L263I, A623R, I431L, S769A/N), and these parasites also harbor unique mutations in \( PfCYTb \) (Y268E, M270S). Given the molecular barcode data these observations suggest that the original C2A strain was a mixture of closely related parasite genotypes from which at least two were selected during subsequent \( Aotus \) passages (represented by passages II/III and passages IV-X, respectively) and another genotype was selected during \( in vitro \) adaptation (TM90C2A).

Serial passaging of C2A in \( Aotus \) selects for increased growth phenotype. As in other biological systems \( P. falciparum \) adaptation (\( in vitro \) and in animals) selects for the genotypes with maximal growth in the absence of any other selection (i.e., drug pressure)\(^27-29\). Increased growth rates typically result in decreased drug susceptibility profiles as demonstrated in the rodent malaria model\(^30\). We therefore wanted to quantify the altered parasite growth rates across \( Aotus \) passages, as a possible contributor to the observed changes in drug susceptibility. We analyzed growth rates during \( Aotus \) adaptation based on the recorded parasitemia from each of the 10 passage experiments (Fig. 4A), considering the time (Fig. 4B) and peak parasitemia (Fig. 4C). Specifically we estimated parasite multiplication rate (PMR, Fig. 4D) from the median of all increases in parasitemia at 48-hour intervals averaged across monkeys in the same passage. These data demonstrated that parasite growth increased significantly from a PMR just above 1 in passage I to PMR of above 7 in passage X. Interestingly increased growth rates were not linked to genomic deletions on chromosomes 2 and 9 (Fig. S1) that occur frequently during \( in vitro \) parasite adaptation\(^31\) and have been observed phenotypically in a splenectomized monkey model\(^32\).

Discussion

In a previous study we observed MQ and AS treatment failures with the Thai multi-drug resistant \( PfC2A \) in \( Aotus \) monkeys\(^33\). Here we systematically investigate the antimalarial drug responses of this isolate and its changes during host adaptation.

Artemisinin resistance in humans is defined as reduced parasite clearance rate or persistence of microscopically detectable parasites on the third day of ACT therapy\(^33\). We observed suppression and persistence of
microscopically detectable parasites beyond the third day of oral AS alone or in combination with MQ, indicating resistance of the Aotus-adapted PfC2A isolate based on the above definition. The observed phenotype is unlikely due to limited AS and/or MQ bioavailability by the oral delivery route as our previous experiments in Aotus with the FVO P. falciparum strain have efficiently cleared parasites at day 4 when AS was administered i.v. or orally at 8 mg/Kg for three days. Similarly MQ treatment as a single dose of 20 mg/Kg cleared parasites on day 5 following initiation of treatment in Aotus infected with FVO. However, we cannot exclude that an inoculum effect may have contributed to the observed relative lower efficiency of the oral route at high parasitemia densities (≥100 × 10^3/μL) compared to the i.v. route at low parasitemia (<1.0 × 10^3/μL). Finally, splenectomy may have resulted in reduced efficiency of artemisinin-mediated parasite clearance as discussed previously.

In vitro and ex vivo experiments demonstrated reduced DHA susceptibility at later Aotus passages, although remaining below the standard resistance threshold of 1% ring stage survival. Genotyping experiments demonstrated presence of wild type Kelch K13 locus across all C2A passages, therefore ruling out involvement of this major resistance locus in the observed in vivo and in vitro or ex vivo drug susceptibility phenotypes. We also demonstrated treatment failure upon MQ treatment in Aotus and MQ drug resistant phenotype in vitro and ex vivo. Pfmdr1 copy number fluctuated between 2 to 2.5 during Aotus adaptation while MQ susceptibility decreased at later passages. These observations contrast with in vitro studies where pfmdr1 de-amplification results in increased MQ susceptibility. For example, in an artelnicic acid (AL)-resistant line of P. falciparum (W2AL80) and clones originating from it, pfmdr1 de-amplification resulted in partial reversal of resistance to AL and increased susceptibility to MQ, even in the absence of drug pressure.

It is possible that both, reduced artemisinin susceptibility and MQ resistance during Aotus adaptation are due to increased growth rates. For example, treatment of neurosyphilis with the zoonotic malaria parasite P. knowlesi was eventually abandoned due to increased virulence of this species after multiple passages in humans. We modeled PfC2A growth rates during Aotus adaptation and indeed demonstrated a significant increase in parasite...
multiplication rate from near 1 (passage I) to above 7 (passage X). It is therefore possible that the increased growth (i.e., parasite fitness) during serial passaging in Aotus resulted in altered drug susceptibility phenotypes. Similar observations have been made in the mouse malaria model, where increased fitness in P. chabaudi correlated with reduced susceptibility to artemisinin.

In conclusion, we present a comprehensive study on Aotus adaptation of the multidrug resistant P. falciparum C2A strain. Combined phenotypic and molecular analysis of parasites across Aotus passages demonstrates that host adaptation has co-selected for reduced drug susceptibility in this non-human primate model. Our data also suggest that this model may be used for the evaluation of anti-malarial drugs against the recently detected Artemisinin resistant strains emerging from South-East Asia.

Materials and Methods

Plasmodium falciparum parasite strains. The original P. falciparum TM90C2A strain and subsequent passages in Aotus (Fig. 5), as well as ex vivo cultures thereof, were used in this study. In addition, the Aotus adapted strain P. falciparum FVO and in vitro adapted strains, D6, D10, CS2 and W2mef, were used as control parasites for these studies.

Animals. Monkeys of the species Aotus l. lemurinus (Panamanian Owl monkey) Karyotype VIII & IX were used in this study. Animals were housed at Gorgas Memorial Institute of Health Studies (ICGES) in Panama, and cared and maintained as described. Briefly, the animals were kept in stainless steel 4 unit quads cages (Lab Products Inc., Seaford, DE) with dimensions of 27 × 23.5 × 29.5 inches. Each cage was fitted with a 3/4-inch-diameter PVC pipe perch placed across 2/3 of the length of the cage and a 6-inch-diameter × 14.5 inches long PVC T pipe nest-box attached to the roof and back of the cage with cable zip ties. Cages were routinely cleaned and sterilized at 180° F at weekly intervals in a cage washing machine (Steris®, Erie, PA). During experimental infections the animals did not receive analgesics.

The experimental protocol was approved by the ICGES Institutional Laboratory Animal Care and Use Committee (CIUCAL) in accordance with procedures described in the "Guide for the Care and Use of Laboratory Animals," 1996; protocol approval number 2006/02. All experiments described herein were performed in accordance with the approved guidelines.

Drug efficacy studies in Aotus monkeys. To determine drug efficacy using the commonly used delivery route in humans, MQ and AS were applied orally alone or in combination. Six splenectomized male and female laboratory bred Aotus weighing between 758–829 g and one spleen intact control were inoculated intravenously (i.v.) in the saphenous vein with 5 × 10⁷ parasitized erythrocytes of the C2A strain from a donor monkey (passage IX). The animals were splenectomized ~30 days prior to inoculation as described. Briefly, the animals were kept in stainless steel 4 unit quads cages (Lab Products Inc., Seaford, DE) with dimensions of 27 × 23.5 × 29.5 inches. Each cage was fitted with a 3/4-inch-diameter PVC pipe perch placed across 2/3 of the length of the cage and a 6-inch-diameter × 14.5 inches long PVC T pipe nest-box attached to the roof and back of the cage with cable zip ties. Cages were routinely cleaned and sterilized at 180° F at weekly intervals in a cage washing machine (Steris®, Erie, PA). During experimental infections the animals did not receive analgesics.

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Before initiation of treatment, citrated whole blood was collected from each animal, plasma removed, and cryopreserved with Glycerolyte® in liquid nitrogen and labeled passage level X for further in vitro IC_{50} determination, Ring Susceptibility Assay (RSA) and genotypic studies. Rescue treatment with MQ at 40mg/Kg orally once and AS at 20mg/Kg i.v. for three days was triggered by any of the following: Hematocrit (HTO) below 50% of baseline, thrombocytopenia (< 50 × 10^3/μL) or signs of depression or anorexia as determined by the attending veterinarian. Two animals were also included in this study as historical references representing infections with C2A passages III (MN24058) and IV (MN26008) shown in Fig. 5 and Table S1.

**In vitro drug assays.** In vitro IC_{50} values were obtained using the hypoxanthine incorporation assay^46. IC_{50} cutoff values indicative of resistance were adopted from the literature as follows: > 100 nM for CQ^21-23,47, 30 nM for MQ^21,22,47, 20 nM for AS^21,23, and 12 nM for DHA^21. Resistance cutoff points for QHS and ATV were not available from the literature. Ex vivo parasite cultures were carried out for up to 8 serial passages in RPMI media containing 25% human AB serum and 4% packed red blood cells. Thin smears were stained with Giemsa for morphological studies and detection of gametocytes. In vitro ring stage 0–3 hours survival assays (RSA) were carried out as described^21 and the threshold for resistance was established at 1% survival.

**Genetic analysis of parasite strains.** Molecular barcode and drug resistance genotyping. Genomic DNA was obtained from blood samples using phenol extraction-ethanol precipitation protocol for purification and concentration of DNA as described^48. DNA concentrations of the samples were measured using a NanoDrop® spectrophotometer ND1000. Extracted DNA was pre-amplified and genotyped across 24 genomic loci with both high-resolution melting (HRM) and TaqMan technologies (Life Technologies, Grand Island, NY) as previously described^25,49. Samples were diluted 1:20 and 5μL of each pre-amplified product used for the molecular barcode. TaqMan barcoding assays were run on a Viia system (Applied Biosystems) and genotypes called using the pre-installed analysis software. To confirm haplotypes and determine drug resistance genotypes of the C2A Aotus passages, we used a high resolution melting (HRM) analysis as described^50.

**Pfmdr1 copy number variation (CNV).** To determine CNV in the pfmdr1 gene across C2A Aotus-adapted passages, gDNA was extracted and used in a qPCR assay, as described^51. Pfmdr1 fold changes between C2A passage levels III-X and reference strain FVO were calculated as described^51. Fold change results were rounded to the next significant integer for the purpose of determining copy number. Primer sequences are included in Table S2.

**Gene sequencing.** To determine single nucleotide polymorphisms (SNPs) in the K13 propeller domain, PCR sequencing was carried out. The full ORF of K13 was PCR amplified using Phusion HF DNA Polymerase kit and primers 1F, 1R. The resulting PCR product of ~2.2kb was purified by gel extraction (QIAquick Gel Extraction Kit, Qiagen) and sequenced at Genewiz using primers 1F, 2F, 3F, 1R, 2R and 3R. Primer sequences are included in Table S2; primers for KAHRP amplification were published previously^52.

**Chromosomal deletions.** To determine whether common genomic deletions in the subtelomeric regions of either chromosome 2 or 9 occurred during C2A passages in Aotus, several loci were analyzed by PCR. For this purpose genomic DNA was extracted using Qiagen™ DNA kit across gDNA was extracted and used in a qPCR assay, as described^13. PCR amplification of genes PFD37_0201500 (PFBO0075c) and PFD37_0202000 (PFBO1000c) on chromosome 2, as well as PFD37_093400 (PFI1710w), PFD37_093600 (PFI1755c), PFD37_093680 (PFI1780w) on chromosome 9 were carried out in a BioRad™ PCR machine using the following amplification program: 95°C (C) × 5 minutes, 95°C × 30 minutes, 51.4°C × 30 minutes, 61.0°C × 3 minutes for 35 cycles, plus 72°C × 10 minutes. Amplicons were loaded onto a 1% agarose gel and subjected to electrophoresis at 150 Volts for 30 minutes, stained with ethidium bromide and photo-documentation done with a UV light reader. Primer sequences are included in Table S2; primers for KAHRP (PFBO1000c) amplification were published previously^51.

**Statistical analysis.** Prism 5 Graph Pad® Software was used to plot and calculate the mean and standard error of the mean (SEM), and the JMP® Pro 10.0.0 statistical program (SAS Institute Inc, Middleton, MA) was used for the Mann-Whitney U significance test between unpaired IC_{50} values for TM90C2A and passage levels II and X. Matlab R2013a was used to plot parasitemia curves and calculate parasite multiplication rate (PMR). PMR is modeled via polynomial regression using the polynomial degree corresponding to the lowest Aikake Information Criteria.

**References**

1. Alonso, P. L. et al. A research agenda to underpin malaria eradication. PLoS medicine 8, e1000406, doi: 10.1371/journal.pmed.1000406 (2011).
2. Sibley, C. H. Understanding artemisinin resistance. Science 347, 373–374 (2015).
3. Price, R. N. et al. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet 364, 438–447, doi: 10.1016/S0140-6736(04)16767-6 (2004).
4. Dondorp, A. M. et al. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 361, 455–467, doi: 10.1056/NEJMoa080839 (2009).
5. Takala-Harrison, S. et al. Independent Emergence of Artemisinin Resistance Mutations Among Plasmodium falciparum in Southeast Asia. J Infect Dis, doi: 10.1093/infdis/jiu91 (2014).
6. Ariey, F. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 450, 50–55, doi: 10.1038/nature22786 (2014).
7. Miotto, O. et al. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat Genet, doi: 10.1038/ng.3189 (2015).
8. Amaratunga, C. et al. Plasmodium falciparum founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. Antimicrob Agents Chemother 58, 4935–4937, doi: 10.1128/AAC.03055-14 (2014).
49. Mharakurwa, S. et al. Pre-amplification methods for tracking low-grade Plasmodium falciparum populations during scaled-up interventions in Southern Zambia. *Malar J* 13, 89, doi: 10.1186/1475-2875-13-89 (2014).

50. Daniels, R. et al. Rapid, field-deployable method for genotyping and discovery of single-nucleotide polymorphisms associated with drug resistance in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 56, 2976–2986, doi: 10.1128/AAC.05737-11 (2012).

51. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT) Method. *Methods* 25, 402–408, doi: 10.1006/meth.2001.1262 (2001).

52. Marti, M., Good, R. T., Rug, M., Knuepfer, E. & Cowman, A. F. Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* 306, 1930–1933 (2004).

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**Author Contributions**

N.O., G.S.D., W.O. and D.K. planned and executed *Aotus* experiments; N.O., M.M., W.O. and G.O.D. collected and analyzed *Aotus* monkey experimental data. N.O., L.G., S.K.V., A.M. and M.M. planned, executed and analyzed *in vitro* drug susceptibility assays. N.O., S.K.V., M.M., R.D., N.B., A.M. and P.Y.M. planned, executed and analyzed molecular biology and genotyping studies. N.O., L.M.C., C.B. and M.M. planned, analyzed and developed growth phenotype models. N.O., M.M., S.K.V., M.T.D. and D.F.W. designed the study. N.O. and M.M. wrote the paper with input from all coauthors.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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