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MECHANISMS OF RESISTANCE

Modification of *pfap2μ* and *pfubp1* Markedly Reduces Ring-Stage Susceptibility of *Plasmodium falciparum* to Artemisinin *In Vitro*

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ABSTRACT Management of uncomplicated malaria worldwide is threatened by the emergence in Asia of *Plasmodium falciparum* carrying variants of the *pfk13* locus and exhibiting reduced susceptibility to artemisinin. Mutations in two other genes, *ubp1* and *ap2μ*, are associated with artemisinin resistance in rodent malaria and with clinical failure of combination therapy in African malaria patients. Transgenic *P. falciparum* clones, each carrying orthologues of mutations in *pfap2μ* and *pfubp1* associated with artemisinin resistance in *Plasmodium chabaudi*, were derived by Cas9 gene editing. Susceptibility to artemisinin and other antimalarial drugs was determined. Following exposure to 700 nM dihydroartemisinin in the ring-stage survival assay, we found strong evidence that transgenic parasites expressing the I592T variant (11% survival), but not the S160N variant (1% survival), of the AP2μ adaptin subunit were significantly less susceptible than the parental wild-type parasite population. The V3275F variant of UBP1, but not the V3306F variant, also displayed reduced susceptibility to dihydroartemisinin (8.5% survival versus 0.5% survival). AP2μ and UBP1 variants did not elicit reduced susceptibility to 48 h of exposure to artemisinin or to other antimalarial drugs. Therefore, variants of the AP2 adaptor complex μ-subunit and of the ubiquitin hydrolase UBP1 reduce *in vitro* artemisinin susceptibility at the early ring stage in *P. falciparum*. These findings confirm the existence of multiple pathways to perturbation of either the mode of action of artemisinin, the parasite's adaptive mechanisms of resistance, or both. The cellular role of UBP1 and AP2μ in *Plasmodium* parasites should now be elucidated.

KEYWORDS *Plasmodium falciparum*, antimalarial agents, gene editing, susceptibility

Artemisinin (ART) combination therapies (ACTs) remain the recommended treatment for uncomplicated *Plasmodium falciparum* infection worldwide (1). Although ACTs are still broadly effective in sub-Saharan Africa, reduced susceptibility to artemisinin and the partner drug piperaquine has been demonstrated throughout the Greater Mekong subregion, and there is some evidence of decreasing effectiveness in Africa (2–9). Reduced susceptibility to artemisinin in Southeast Asia has been linked to particular mutations in the propeller domain of the *pfkelch13* (K13) gene (2, 3). However, these mutations are not widely observed in other regions, and the distinct K13 mutations identified at a low frequency in Africa do not correlate with either delayed clearance or parasite recrudescence in field studies and travelers (6, 8, 9). Reduced artemisinin susceptibility *in vitro* can be generated experimentally by the introduction of variant K13 genes into transgenic parasite lines (10), but an understanding of other genetic determinants of artemisinin and partner drug failure is critical for preserving ACT efficacy worldwide.

In 2007, Hunt et al. evolved artemisinin-resistant lines of the rodent malaria parasite *Plasmodium chabaudi in vivo* and described mutations associated with the phenotype

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by linkage analysis (11). These included variants of *pcubp1*, encoding a putative ubiquitin hydrolase, and *pcap2μ*, encoding the μ-subunit of the AP2 vesicular trafficking complex (11, 12). Subsequent gene sequencing of peripheral blood parasites from Kenyan patients revealed that the Ser160Asn mutation in the *P. falciparum* homologue of this gene, *pfap2μ*, was associated with ACT treatment failure and that certain alleles of *pfubp1* were also more common among parasites surviving treatment (13). Similar alleles of *pfubp1* had been previously identified in studies of *in vitro* artemisinin susceptibility of cultured Kenyan isolates of *P. falciparum* (14). We generated transgenic parasites *in vitro* that harbored an additional, constitutively expressed copy of the modified locus PfAP2$\mu^{Ser160Asn}$, a genotype which currently circulates across Africa (8, 13). Some evidence was found of altered susceptibility to artemisinin and other frontline chemotherapies in these lines, in which the endogenous wild-type locus was also present and active (15). The availability of genome-editing systems in *Plasmodium falciparum* now provides an opportunity to perform gene replacement of the endogenous loci of interest to more directly test the impacts of *pfap2μ* and *pfubp1* mutations on parasite drug susceptibility (10).

In this study, we used Cas9 editing to generate parasites harboring the PfAP2$\mu^{Ile592Thr}$ orthologue of the PcAP2$\mu^{Ile568Thr}$ *P. chabaudi* mutation (12) and the PfAP2$\mu^{Ser160Asn}$ variant seen in Africa (13). We also introduced into the *pfubp1* locus, separately, sequences encoding the Val3275Phe and Val3306Phe variants, corresponding to *P. chabaudi* mutations previously described by Hunt et al. (11). The *in vitro* susceptibility to artemisinin and other frontline antimalarials of these gene-edited parasites was determined and compared to those of previously validated ART-resistant *P. falciparum* isolates of Cambodian origin expressing K13 variants.

RESULTS

AP2μ modifications alter *in vitro* susceptibility to frontline chemotherapies. The orthologue of the *P. chabaudi* AP2$\mu^{Ile568Thr}$ variant (11), PfAP2$\mu^{Ile592Thr}$, was efficiently installed by CRISPR-Cas9 editing onto the *P. falciparum* 3D7 background. In parallel, a PfAP2$\mu^{Ile592Ile}$ transgenic line expressing the wild-type Ile592 codon in the context of recodonization ablating the Cas9 protospacer adjacent motif (PAM) site was also generated (Fig. 1A and B, left) to rule out a role for silent mutations in drug susceptibility. Both lineages were cloned by limiting dilution, and profiles of susceptibility of two clones of each lineage to chloroquine (CQ), quinine, dihydroartemisinin (DHA), lumefantrine, piperaquine, and mefloquine were characterized. While there was no difference between mutant and wild-type parasites in susceptibility to a 48-h exposure to DHA, quinine, piperaquine, or mefloquine, the 592Thr parasites were more susceptible to lumefantrine (592Thr, 21.3 nM; Ile592, 30.83 nM; mean difference, 9.15 nM [$P = 0.0067$]) and chloroquine (592Thr, 8.05 nM; Ile592, 11.88 nM; mean difference, 3.83 nM [$P < 0.0001$]) (Table 1). No effect on intraerythrocytic growth was seen with the Ile592Thr transgenic lines (see Fig. S1 in the supplemental material).

We then characterized the susceptibility of these clones to artemisinin using the ring-stage survival assay (RSA) (2, 16), an *in vitro* correlate of *in vivo* parasite susceptibility in which early-ring-stage parasites are exposed to a brief pulse of 700 nM DHA. Artemisinin susceptibility was also determined for three Cam3.II-derived Cambodian parasite lines (10), and the parental 3D7 line, as validated controls. Both clones encoding the PfAP2$\mu^{Ile592Thr}$ variant displayed higher ring-stage survival rates (clone 1, 11.2% survival; clone 2, 9.3% survival) than the parental 3D7 strain (1.3% survival). Silent recodonization had no effect on artemisinin susceptibility, as both gene-edited Ile592Ile clones displayed artemisinin susceptibility equivalent to that of 3D7 (Fig. 2). For comparison, the Cam3.II Cambodian clinical isolate harboring the PfK13^{Arg539Thr} variant and a derived gene-edited line harboring PfK13^{Cys580Tyr} displayed significantly enhanced survival rates in the RSA of 28.0% and 17.3%, respectively, compared to a gene-edited Cam3.II line expressing wild-type K13 (Cam3.II^{REV}; 2.63% survival).

Clones of a lineage expressing PfAP2$\mu^{Ser160Asn}$ (Fig. 1A and B, right), which currently circulates in Africa and was previously associated with delayed parasite clearance in

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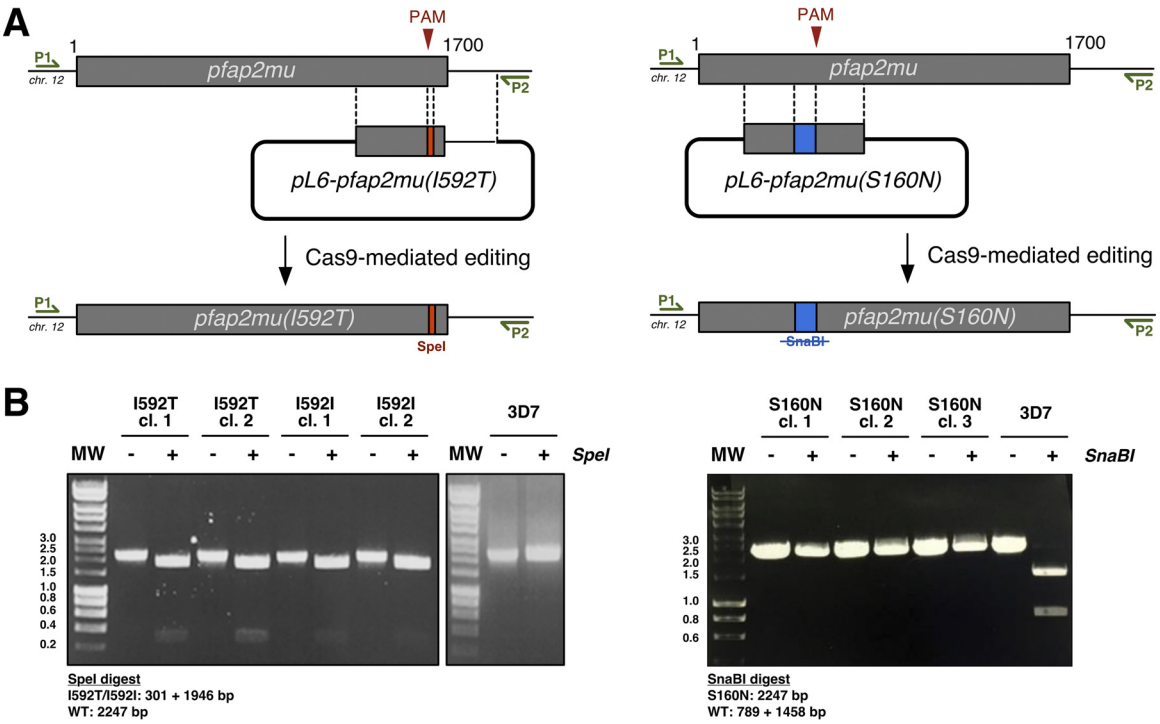

FIG 1 Gene editing of *pfap2*μ. (A) CRISPR-Cas9 editing was used to install the *pfap2*μ^{I592T} variant codon (red) (left) and the *pfap2*μ^{S160N} variant codon (blue) (right) into the endogenous locus. The pictured homologous repair constructs were modified to also introduce the I592I wild-type codon in the context of silent mutations ablating the Cas9 PAM site, as a control for their impact on phenotype. Primers used for genotype mapping anneal outside the homologous repair template, as depicted (P1 and P2) (see Table S1 in the supplemental material). The recodonized sequence near alternate I592 codons (red) also includes a SpeI restriction site, and the recodonized sequence near S160 codons (blue) ablates an SnaBI restriction site for downstream PCR-RFLP mapping. (B) Clones of parasite lines expressing AP2μ^{I592T} and AP2μ^{I592I} (left) and AP2μ^{S160N} (right) were generated by limiting dilution and confirmed by PCR-RFLP genotype mapping (with SpeI or SnaBI restriction endonuclease) and Sanger sequencing. Amplification of the locus with P1 and P2 produces a 2,247-bp fragment (Table S1); for I592I transgenic parasites, SpeI digestion of an amplicon containing the transgenic locus liberates a 301-bp fragment. The native amplicon is not cleaved (3D7 lanes). For S160 transgenic parasites, SnaBI does not cleave the transgenic amplicon, while wild-type (WT) amplicons liberate a 789-bp fragment. MW, fragment length in kilobases.

clinical studies, did not show enhanced survival in this assay (Fig. 2). This mutant allele also had no effect on 48-h susceptibility to any of the drugs examined (Table 1). These data confirm that a single amino acid change in AP2μ at codon 592 can confer significantly increased ring-stage survival on an otherwise artemisinin-susceptible genetic background.

The Val3275Phe variant of PfUBP1 reduces ring-stage artemisinin susceptibility *in vitro*. To evaluate the impacts of UBP1 mutation on parasite susceptibility to artemisinin and other frontline chemotherapies, we used Cas9 editing to generate two *P. falciparum* lineages expressing orthologues of the original *P. chabaudi* mutations,

TABLE 1 Comparison of the *in vitro* susceptibility of *pfap2*μ variant transgenic parasite lines with that of parental 3D7 exposed to dihydroartemisinin or other antimalarial drugs for 48 h

Compound	Mean EC_{50} ± SEM (nM)[a]						
	3D7 parental	AP2μ^{S160N} clone 1	AP2μ^{S160N} clone 2	AP2μ^{I592I} clone 1	AP2μ^{I592I} clone 2	AP2μ^{I592T} clone 1	AP2μ^{I592T} clone 2
Chloroquine	13.7 ± 0.3	13.5 ± 0.1	14.9 ± 0.4	11.4 ± 0.8	12.3 ± 0.4	**8.7 ± 0.9**	**7.4 ± 0.3**
Quinine	31.4 ± 2.6	27.7 ± 2.0	32.3 ± 3.3	23.4 ± 2.5	24.0 ± 2.7	24.8 ± 1.9	23.0 ± 2.3
Piperaquine	31.6 ± 4.5	28.0 ± 3.1	29.6 ± 5.0	23.5 ± 3.6	25.9 ± 0.7	26.5 ± 3.0	22.8 ± 2.5
Mefloquine	12.2 ± 0.2	12.1 ± 0.5	12.0 ± 0.7	16.8 ± 2.4	17.0 ± 1.4	16.2 ± 1.8	16.0 ± 1.9
Lumefantrine	30.0 ± 4.0	26.9 ± 2.7	29.6 ± 3.0	30.2 ± 3.0	31.4 ± 2.3	**20.9 ± 2.1**	**21.8 ± 1.0**
Dihydroartemisinin	3.4 ± 0.3	2.9 ± 0.2	2.7 ± 0.7	2.8 ± 0.6	3.1 ± 0.7	3.1 ± 0.7	2.5 ± 0.4

[a]EC_{50} values are the means ± SEM from at least four biological replicates (different cultures and different plates), each performed in technical duplicate. Boldface type indicates a *P* value of <0.01 (Mann-Whitney U test), compared to the EC_{50} estimate for the 3D7 parental line.

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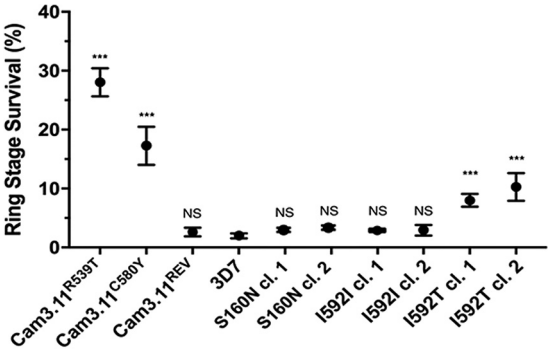

FIG 2 RSA$^{4\ h}$ survival estimates for *pfap2μ* variant transgenic parasite lines (clones 1 and 2) exposed to artemisinin. Shown is RSA$^{4\ h}$ percent survival of mutant and parental parasite lines compared to the untreated control, following a 4-h pulse of 700 nM dihydroartemisinin. The Cam3.II family of parasite lines harbors *pfk13* mutations, as indicated; REV indicates that wild-type K13 is encoded (10). The mean of data from at least four biological replicates is shown for each line, each performed in technical duplicate, with the standard error. Each technical replicate enumerates 100,000 gate-stopping events. *P* values were derived by a Mann-Whitney U test, comparing each parasite line to 3D7. ***, $P < 0.005$; NS, nonsignificant.

encoding PfUBP1^{Val3275Phe} and PfUBP1^{Val3306Phe} (Fig. 3). The donor templates and single guide RNA (sgRNA) Cas9 guide sequences used to generate each mutation were identical except for these point mutations. Three weeks after transfection and selection, transgenic parasites were obtained, cloned by limiting dilution, and genotyped by PCR-restriction fragment length polymorphism (RFLP) mapping and Sanger sequencing.

Two clones of each parasite lineage were examined for their susceptibility to 48-h exposures to dihydroartemisinin, chloroquine, quinine, and piperaquine (Table 2). There were no differences in 50% effective concentration (EC_{50}) estimates for any chemotherapy between either clone of 3D7-UBP1^{Val3275Phe} and 3D7. Both clones of 3D7-UBP1^{Val3306Phe} were more sensitive than 3D7 to chloroquine. There were no differences in EC_{50} estimates for each drug between the clones of each mutant lineage.

We then examined both clones of each of the *pfubp1* mutant lineages for ring-stage susceptibility to dihydroartemisinin in the RSA (Fig. 4). Parasites expressing UBP1^{Val3275Phe} displayed a >5-fold increase in ring-stage survival compared to wild-type 3D7 progenitors (clone 1, 6.46%; clone 2, 6.54%; 3D7, 1.2% [$P < 0.005$]). The two Val3306Phe clones displayed sensitivity equivalent to that of 3D7 (clone 1, 1.46%; clone 2, 1.96% [$P > 0.05$]). There was no difference in survival between the two clones within each mutant lineage, and no effect on intraerythrocytic growth was seen (Fig. S1).

DISCUSSION

In this study, we present evidence that mutations in genes encoding the μ-subunit of the AP2 trafficking complex and the ubiquitin hydrolase UBP1 can generate reduced *P. falciparum* ring-stage artemisinin susceptibility *in vitro* similar to that characteristic of K13-mediated ART resistance circulating in the Greater Mekong subregion (10). These susceptibility phenotypes are also similar to those seen in recently described laboratory-evolved parasites of Senegalese origin harboring PfCoronin variants (17). Thus, reduced *in vitro* ring-stage susceptibility due to a single amino acid substitution has now been demonstrated for four loci, including *pfk13*.

Resistance to artemisinin has long been proposed to be multigenic. So far, laboratory evolution of reduced artemisinin susceptibility has failed to generate parasites with the most prevalent K13 mutations seen in the field, confirming the importance of examining the parasite genetic background when considering drug resistance in the clinic. Resistant variants in K13 occur naturally only in the presence of variants of certain other proteins, perhaps compensating for a loss of fitness due to the primary change (18, 19), and this is supported by the results of a recent genetic cross of parasites harboring *pfk13* C580Y with *pfk13* wild-type parasites (20). Although the *pfk13* geno-

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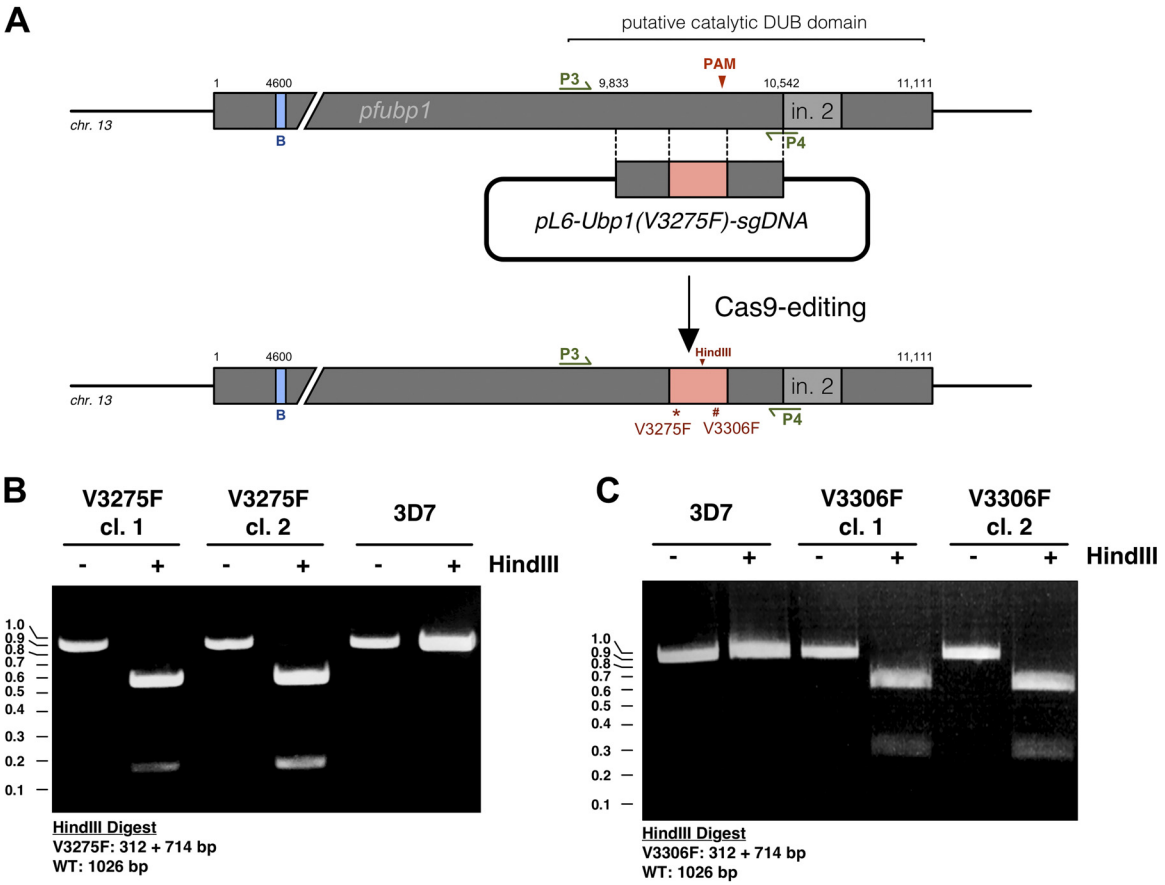

FIG 3 Gene editing of *pfubp1*. (A) CRISPR-Cas9 editing was used to install the *pfubp1*^{Val3275Phe} *P. falciparum* orthologue of the previously described *P. chabaudi* mutation into the endogenous locus in 3D7, marked with an asterisk. The same scheme and homology regions were used to install *pfubp1*^{Val3306Phe}, marked with a number sign. Installation of either mutation introduces a HindIII restriction site, as marked. Primers P3 and P4 (see Table S1 in the supplemental material) were used to perform PCR-RFLP mapping of transgenic clones. B indicates the position of the "Borrmann hot spot" of variation associated with reduced artemisinin susceptibility (13, 14). Coordinates above the gene are in base pairs. "in. 2" denotes the position of the second intron in the gene. (B) Clones of parasite lines expressing UBP1^{Val3275Phe} were generated by limiting dilution and confirmed by PCR-RFLP genotype mapping (with primers P3 and P4 and HindIII restriction digestion in lanes marked with a plus sign) and by Sanger sequencing. (C) Clones of parasite lines expressing UBP1^{Val3306Phe} were generated and confirmed as described above for Val3275Phe mutants.

type at codon 580 determined whether the RSA survival rate was high or low among the progeny, variation within these two groups was modulated by other heritable variant loci. Previous work has implicated intracellular trafficking and protein turnover in the mechanism of artemisinin resistance (21), and our work here provides further support for these conclusions. In nearly all other systems, the AP2 trafficking complex mediates endocytosis from the plasma membrane, and deubiquitinases are often involved in protein degradation and recycling. A functional characterization of both

TABLE 2 Comparison of the *in vitro* susceptibility of *pfubp1* variant transgenic parasite lines with that of parental 3D7 exposed to dihydroartemisinin or other antimalarial drugs for 48 h

Compound	Mean EC_{50} ± SEM (nM)[a]				
	3D7 parental	Ubp1^{V3306F} clone 1	UBP1^{V3306F} clone 2	UBP1^{V3275F} clone 1	UBP1^{V3275F} clone 2
Chloroquine	18.8 ± 1.4	13.6 ± 3.0	13.7 ± 2.6	15.3 ± 1.4	14.2 ± 3.0
Quinine	54.9 ± 12	60.5 ± 15	53.8 ± 15	66.8 ± 21	60.6 ± 17
Piperaquine	14.9 ± 6.4	9.8 ± 3.5	8.4 ± 0.9	13.4 ± 2.0	11.0 ± 3.2
Dihydroartemisinin	4.3 ± 0.8	3.3 ± 0.6	3.6 ± 0.6	4.1 ± 0.7	3.8 ± 0.5

[a]See the footnote to Table 1.

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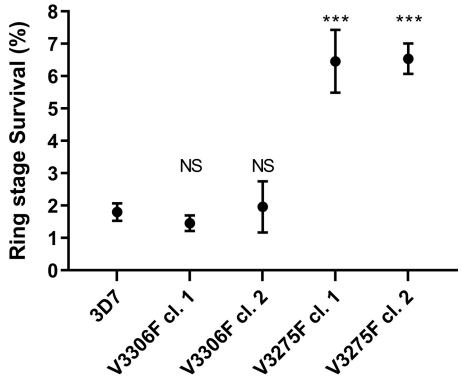

FIG 4 RSA$^{4\ h}$ survival estimates for *pfubp1* variant transgenic parasite lines (clones 1 and 2) exposed to artemisinin. RSA survival of mutant and parental parasite lines was compared to that of the untreated control, following a 4-h pulse of 700 nM dihydroartemisinin. The mean of data from at least four biological replicates is shown for each line, each performed in technical duplicate, with the standard error. Each technical replicate enumerates 100,000 gate-stopping events. *P* values were derived by using the Mann-Whitney U test, comparing each parasite line to 3D7. ***, $P < 0.005$; NS, nonsignificant.

proteins is needed to resolve the broader mechanisms underlying this complicated phenotype.

Our findings clarify that the PfAP2$\mu^{Ser160Asn}$ mutation has no effect on ring-stage artemisinin susceptibility. Our previous conclusions suggested a possible increase in the DHA 48-h EC_{50} when overexpressing Ser160Asn in a multidrug-resistant background (the Dd2 parasite line from Southeast Asia) (15). However, even though this work was performed before the standardization of the RSA, we found no evidence of increased survival to a ring-stage artemisinin pulse at that time. Here, using the RSA and 48-h EC_{50} assay formats, we also failed to detect any reduced artemisinin susceptibility mediated by this mutation in a chloroquine-sensitive parasite background (3D7). Further Cas9-editing experiments should be done in the CQ-resistant Dd2 background, but our results to date suggest that Ser160Asn is insufficient to generate reduced artemisinin susceptibility alone, and thus, any correlation with ACT failure observed in human infections (8, 13, 15) is due to either other benefits of the genotype or linkage with other genetic factors. Nevertheless, our RSA results from the Ile592Thr mutant clearly indicate that AP2μ variants can elicit reduced ring-stage parasite susceptibility to artemisinin.

Our data also confirm the ability of variants of UBP1 to mediate reduced artemisinin susceptibility. We examined the UBP1 and AP2μ mutations identified in *P. chabaudi* in isolation to understand their individual contributions to artemisinin sensitivity. In the rodent model, the orthologue of PfUBP1^{Val3306Phe} cooccurred with the orthologue of PfAP2$\mu^{Ile592Thr}$, whereas the latter arose specifically during artemisinin pressure (11). Consistent with this, in our hands, PfUBP1^{Val3306Phe} is insufficient to generate artemisinin resistance. An obvious next experiment is to examine the effects of these mutations together in a single lineage. Interestingly, neither *pfubp1* mutation conferred any protection from chloroquine, as the rodent model might suggest. However, careful analysis of the linkage data from the seminal experiments of Hunt et al. revealed that *pcubp1* mutations were associated with CQ resistance only in the context of another mutation in *pcaat1*, an amino acid transporter (11). As with AP2μ, exploring these combinatorial effects on drug susceptibility in *P. falciparum* will form the basis for important future studies.

The RSA as an *in vitro* test of artemisinin susceptibility phenotype is only a few years old and has been developed in parallel in different laboratories since it was first described (22). Interlaboratory variability has not yet been explored with any rigor, but as an initial approach to ensuring comparability among results obtained from different research teams, the inclusion of common control parasite lines is an important step toward standardization. We were able to use the Cam3.II series of *P. falciparum* lines

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engineered in the Fidock laboratory as controls in our RSAs and obtained survival estimates for each that were broadly in line with those reported in the original work of Straimer et al. describing them (10). On this basis, we can confidently compare RSA survival estimates for our transgenic mutant lines back to those obtained for the 3D7 parental line. However, as laboratories differ in the duration of the 700 nM DHA pulse used, and in the methodology for survival readout (flow cytometry or microscopy) (16, 17), further work on assay standardization is required before interlaboratory comparisons of survival estimates will have adequate validity. This is likely to include simple normalization approaches, based on relative survival compared to agreed standard comparators.

Resistance to artemisinins is unlike that of any other class of antimalarial compounds, being confined to the first few hours of intraerythrocytic development. We and others have now demonstrated this phenomenon with mutations in *pfk13* (22), *pfcoronin* (17), *pfap2μ*, and *pfubp1* as well as with transient pulses of low temperature (16), suggesting that artemisinin resistance has potentially many genetic and environmental origins. In all these cases so far, trophozoites and schizonts are fully sensitive to artemisinin. Furthermore, even "resistant" ring-stage parasite forms are still mostly sensitive (e.g., more than 70% of parasites are killed by a single 700 nM DHA exposure). It follows that, eventually, even mutant parasite populations should be cleared *in vivo* by extended artemisinin regimens or next-generation analogues with longer half-lives (23). This pattern also suggests that there is a particular set of cellular processes or circumstances in those first hours of development that are amenable to manipulation by mutation, leading to protection from artemisinin's oxidative mechanism of action. Recent studies and our data here suggest that these processes likely involve intracellular traffic and protein turnover, two fundamental aspects of parasite biology. Given the success of artemisinins globally, investigations into these mechanisms may reveal other loci capable of manipulating parasite artemisinin sensitivity. Potentially, some of these may be of relevance to African settings, where K13 variants have not yet emerged as a cause of treatment failure. Although studies to date in the Mekong region have not described any polymorphisms in the *pfap2μ* locus, this gene has now been identified as lying in a region of chromosome 12 showing evidence of recent strong selection in Ghana and Malawi (24). Conversely, a mutation in *pfubp1* at codon 3138 was found to be associated with *P. falciparum* isolates from Thai malaria patients carrying the C580Y mutation in *pfk13* and has thus been implicated in slow clearance after artemisinin therapy (19). Given that reduced ring-stage artemisinin susceptibility *in vitro* is now confirmed to have multiple potential origins, tracking the phenotypes of delayed parasite clearance and early recrudescence may be more effective than surveillance of specific mutations in African *P. falciparum* populations.

MATERIALS AND METHODS

Plasmid design and construction. Plasmids pL6-AP2$\mu^{Ile592Thr}$-sgDNA and pL6-AP2$\mu^{Ile592Ile}$-sgDNA, including a recodonized donor sequence carrying the mutation of interest, were constructed as described previously (25). Constructs to install the *pfap2$\mu^{Ser160Asn}$*, *pfubp1^{Val3275Phe}*, and *pfubp1^{Val3306Phe}* mutations were similarly designed (see the supplemental material).

Parasite lines. Plasmid constructs were transfected into laboratory clone 3D7. Cambodian-origin lines Cam3.II^{R539T}, Cam3.II^{C580Y}, and Cam3.II^{REV} were kindly provided by the laboratory of David Fidock (10). All Cam3.II lineage parasites harbor wild-type *pfap2μ* and *pfubp1* alleles.

Parasite culture and generation of transgenic parasites. *Plasmodium falciparum* culture was performed in A$^+$ erythrocytes obtained from the UK Blood Bank. Parasites were cultured in RPMI supplemented with AlbuMAX II, L-glutamine, and gentamicin, at 5% hematocrit under 5% CO_2 conditions at 37°C. Parasites were synchronized using 70% Percoll gradients to capture schizonts and 5% sorbitol to isolate ring-stage parasites.

For transfection, 3D7 parasites were cultured to approximately 10 to 15% ring-stage parasitemia at 5% hematocrit under standard conditions. Immediately before transfection, 100 μg of each plasmid was resuspended in 100 μl of sterile Tris-EDTA (TE) buffer. Two hundred fifty microliters of packed cells equilibrated in 1× Cytomix (120 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES, 0.15 mM $CaCl_2$, 2 mM EGTA, 10 mM KH_2PO_4-K_2HPO_4 [pH 7.6]) was combined with 250 μl of 1× Cytomix in a 2-mm transfection cuvette (Bio-Rad Laboratories), and plasmid DNA was added. The cells were pulsed at 310 V, 950 μF, and infinite resistance in a Bio-Rad gene pulser; washed; and returned to culture. Fresh erythrocytes were added to approximately 5% total hematocrit on day 1 after transfection, along with 2.5 nM WR99210 and 1.5 μM

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DSM-1. Media and selection drugs were replenished every day for 14 days and then every 3 days until parasites were observed by microscopy. Parasites recovered to microscopically detectable levels at approximately 3 weeks posttransfection, and the genotype was confirmed by PCR-RFLP mapping and Sanger sequencing. In particular, the donor template for installing *pfap2μ*(*I592T*) introduced a silent SpeI restriction site, and the template for installing *pfap2μ*(*S160N*) silently ablated an endogenous SnaBI restriction site. Both donor templates for *pfubp1* mutations introduced HindIII restriction sites. Parasites were cloned by limiting dilution, whereby cultures were seeded at 0.3 parasites/well in 250 μl of a 2% hematocrit culture in a 96-well plate. Spent medium was removed and replaced with complete medium supplemented with 0.5% (vol/vol) fresh red blood cells weekly for 3 weeks. Parasite growth was examined after 3 weeks by a lactate dehydrogenase (LDH) assay and microscopy, and several clones were selected for genotype confirmation and further characterization. Approximately one-third of each cloning plate contained parasites, consistent with the seeding parasite density.

***In vitro* drug susceptibility assays.** Synchronized ring-stage parasites at 0.5% parasitemia and 2% hematocrit were exposed to dihydroartemisinin (DHA), quinine, chloroquine, lumefantrine, piperaquine, and mefloquine over a full 48-h life cycle in two biological replicates in a 96-well array bearing serial 2-fold drug dilutions. The plate was incubated at 37°C for 48 h, and EC_{50} estimates were determined as described previously (26). Assays to measure artemisinin survival of synchronized ring-stage parasites within 4 h of erythrocyte invasion ($RSA^{0-4\ h}$) were carried out exactly as described previously (16). Fluorescence-activated cell sorter (FACS) analysis was performed on live cells stained with a 1:10,000 dilution of MitoTracker deep red and a 1:1,000 dilution of SYBR green (Invitrogen-Molecular Probes) in phosphate-buffered saline (PBS) for 20 min at 37°C. Cells were stained using 8 volumes of stain solution per volume of culture (2% hematocrit) and analyzed using an LSR-II flow cytometer (BD Biosciences). Statistical comparisons of drug susceptibility were performed by using the nonparametric Mann-Whitney U test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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R.C.H. conceived, designed, and executed the study. R.C.H. performed cell culture, transfections, and drug susceptibility assays. D.A.V.S. planned experiments and performed cell culture and drug susceptibility assays. C.J.S. conceived, designed, and supported the study. R.C.H. and C.J.S. wrote the paper, with input from D.A.V.S.

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