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Lack of Association of the S769N Mutation in *Plasmodium falciparum* SERCA (PfATP6) with Resistance to Artemisinins

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The recent emergence of artemisinin (ART) resistance in *Plasmodium falciparum* in western Cambodia, manifested as delayed parasite clearance, is a big threat to the long-term efficacy of this family of antimalarial drugs. Among the multiple candidate genes associated with ART resistance in *P. falciparum*, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase PfATP6 has been postulated as a specific target of ARTs. The *PfATP6* gene harbors multiple single-nucleotide polymorphisms in field parasite populations, and S769N has been associated with decreased sensitivity to artemether in parasite populations from French Guiana. In this study, we used an allelic exchange strategy to engineer parasite lines carrying the S769N mutations in *P. falciparum* strain 3D7 and evaluated whether introduction of this mutation modulated parasite sensitivity to ART derivatives. Using three transgenic lines carrying the 769N mutation and two transgenic lines carrying the wild-type 769S as controls, we found that S769N did not affect *PfATP6* gene expression. We compared the sensitivities of these parasite lines to three ART derivatives, artemether, artesunate, and dihydroartemisinin, in 18 biological experiments and detected no significant effect of the S769N mutation on parasite response to these ART derivatives. This study provides further evidence for the lack of association of PfATP6 with ART resistance.

Artemisinin (ART) and its derivatives play an indispensable role in the malaria elimination/eradication campaigns currently being unfolded in many regions where malaria is endemic. To reduce the chance of resistance development and prolong the life span of this group of drugs, the World Health Organization (WHO) has endorsed ART-based combination therapies (ACTs) as the first-line treatment for *Plasmodium falciparum* malaria (47). Since the adoption of the ACT policy in many regions where *P. falciparum* malaria is endemic (5), a trend of steady reduction in global malaria incidence has been observed (61). However, the recent detection of emerging low-grade resistance to ARTs in western Cambodia, manifested as delayed parasite clearance, has raised a major concern (20, 45). The Greater Mekong Subregion (GMS) has been an epicenter of drug resistance, and resistance to chloroquine (CQ) and pyrimethamine has spread from there to Africa (51, 63). Therefore, an analogous spread of ART resistance from this region would be a disaster. As WHO has been gathering resources for eliminating and containing ART-resistant parasites (61), surveillance efforts have intensified in the GMS, where ART use has the longest history. Meanwhile, research aimed to decipher the underlying mechanisms of ART resistance has become a priority.

ARTs contain an endoperoxide bridge that is essential for the parasite-killing activities (60). Although the structure of ART was solved over 3 decades ago, the mode of action of this group of drugs has not been unequivocally determined (16, 19, 48). The most-studied model suggests that heme-mediated activation of ARTs results in C-centered free radicals that alkylate biomolecules in the parasite, leading to parasite death (32, 38, 39). Evidence supporting the involvement of heme in the action of ARTs includes antagonistic actions of iron chelators and the requirement of hemoglobin digestion for the activity of ART (30, 40). This also correlates with the tolerance phenomenon of ring-stage parasites to ARTs, when hemoglobin digestion activity is low. The reduced metabolic activity at the ring stage is reflected further in ART-induced temporary arrest of growth (dormancy) at this stage (10, 62). Whereas this may partially explain the prolonged parasite clearance observed in clinical studies (46), the possibility of host factors that may play a crucial role in determining prolonged parasite clearance times observed *in vivo* has not been investigated (9, 58). In addition, it has been proposed that ARTs may interfere with the mitochondrial function of the parasite (36, 59). Other postulated cellular targets of ARTs include the multidrug resistance 1 (mdr1) gene, ABC transporter genes G7 and G49 (1), translationally controlled tumor protein (4), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ortholog PfATP6 (21). In rodent malaria caused by *Plasmodium chabaudi*, a mutation in the deubiquitinating enzyme ubp-1 has been mapped as a determinant of experimentally selected ART resistance (24). Despite these proposed targets, no definite genetic determinant of *Plasmodium* sensitivity to ARTs has been identified so far. Moreover, none of these candidate genes appears to be responsible for the observed ART resistance in western Cambodia (20, 26).

The proposal of PfATP6 as the primary target of ARTs in malaria parasites was initially based on the structural resemblance of ARTs to thapsigargin, a specific inhibitor of mammalian SERCAs. Since PfATP6 is the only SERCA-type Ca^{2+}-ATPase in the malaria parasite's genome, it was evaluated as the target of ARTs. When expressed in *Xenopus laevis* oocytes, PfATP6 can be specifically inhibited by ART as well as thapsigargin (21). Modeling of PfATP6 and docking simulations suggest that ARTs bind to

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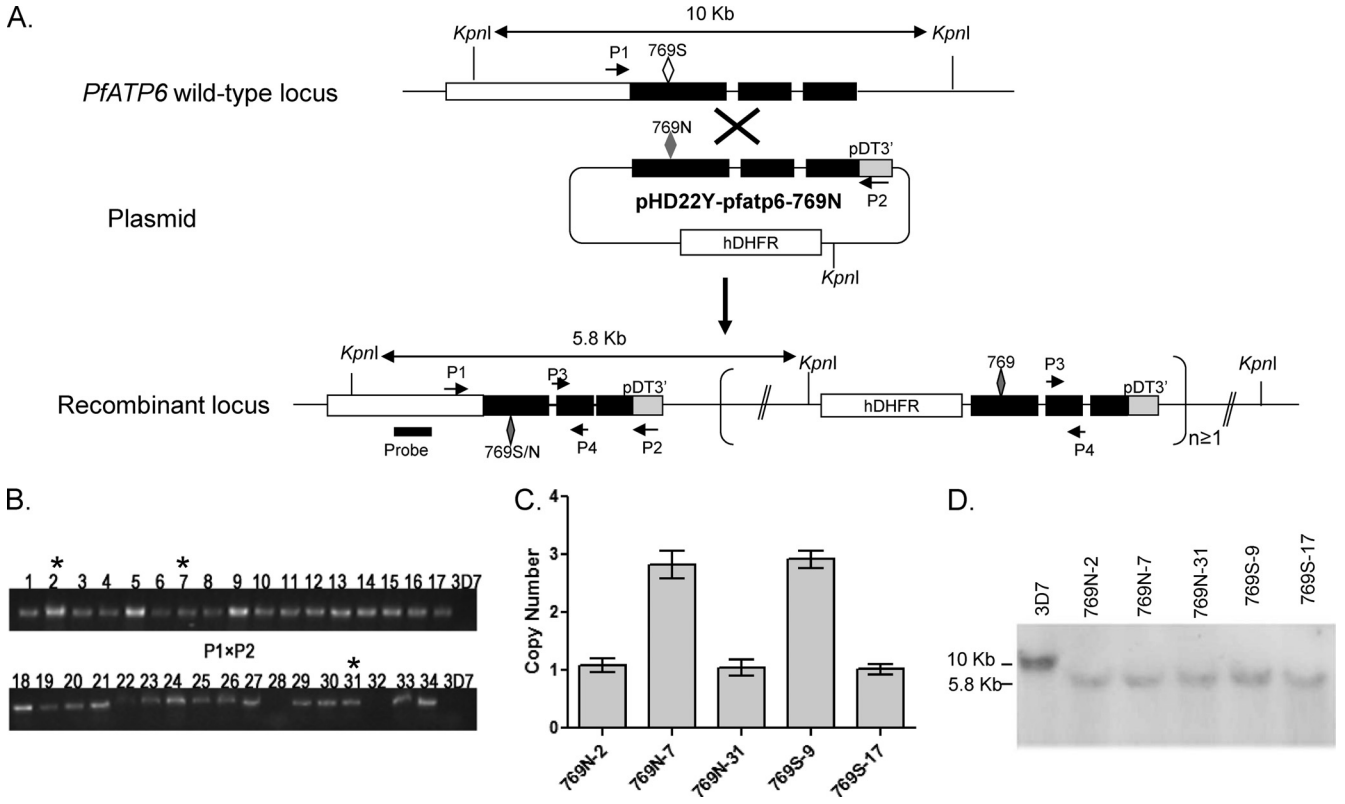

FIG 1 Development of transgenic lines in 3D7 with the PfATP6 S769N mutation. (A) Schematic representation of single-crossover event at the *Pfatp6* locus. (Top) The *Pfatp6* locus on chromosome 1. Solid lines represent introns or intergenic regions, and filled boxes indicate the coding regions. (Middle) Plasmid pHD22y-pfatp6-769N, showing the *Pfatp6* genomic fragment and the drug selection cassette hDHFR. (Bottom) Predicted single-crossover events at the *Pfatp6* locus. The fragment within the bracket indicates the scenario when integration of concatemerized plasmid occurs. The C-terminal fragment of *PfATP6* cloned in the transfection plasmid is shown as filled boxes. The open and filled lozenges indicate the locations of the wild-type and mutant amino acids at position 769, respectively. Restriction enzyme KpnI sites and the expected sizes of DNA fragments after KpnI digestion are illustrated. The positions and orientations of the primers on chromosome 1 and the plasmid are shown. Primer pairs P1 and P2 were used for integration-specific PCR, whereas primers P3 and P4 were used for determining the copy number of the integrated plasmid. The position of the probe used for genomic Southern blotting is also marked. (B) Integration-specific PCR products, based on used of primer P1 on chromosome 1 and primer P2 on the plasmid, showing 32 positive and 2 negative clones (lanes 28 and 32). The PCR products of 32 positive clones were sequenced, and asterisks indicate the three clones with the S769N mutation. (C) Copy numbers of the integrated plasmid or concatemers at the *Pfatp6* locus as determined by real-time PCR using primer pairs P3 and P4. Shown here are five transgenic clones, with two containing the wild-type residue (739S-9 and 769S-17) and three with the 769N mutation (769N-2, 769N-7, and 769N-31). (D) Genomic Southern blot of DNA isolated from 3D7 and five clones with plasmid integration at the *Pfatp6* locus. Genomic DNA was digested with KpnI and separated in a 1% agarose gel. The blot was hybridized with the probe marked in panel A, which revealed a ca. 10-kb fragment in 3D7 and 5.8-kb fragment in the recombinant *Pfatp6* locus.

PfATP6 through hydrophobic interactions (29, 44). Variations at a single residue, 263, located in the predicted ART-binding pocket of PfATP6, tremendously affect the sensitivity of the enzyme to ARTs (56). When assayed in *X. laevis* oocytes, the introduction of a single substitution, L263A or L263S (residues in *Plasmodium vivax* and *Plasmodium berghei* SERCAs, respectively) resulted in an approximately 3-fold increase or decrease of sensitivity to ARTs, respectively. Furthermore, the L263E replacement led to complete abolishment of inhibition by ART (56). However, this observation was not extended to *P. falciparum*, where introduction of the L263E mutation through transgenics resulted in borderline nonsignificant changes in the 50% inhibitory concentrations (IC_{50}s) for ART and its derivatives (57). Recently, Lepore et al. performed modeling and docking simulations for SERCA proteins from *P. falciparum*, *Schistosoma mansoni*, and humans, but they did not find significant differences in the binding mode of artemether (ATM) to these proteins (35). Since the SmSERCA has a 263E residue and ATM still kills *S. mansoni*, it has been argued that the residue at 263 may be less critical. Whereas a mutation at position 263 has not been detected in field isolates from regions with suspected ART resistance, Jambou et al. reported an association of reduced *in vitro* ATM susceptibility with an S769N substitution in a limited number of parasite field isolates from French Guiana (27). Additionally, this mutation was later detected in a few isolates from Senegal, and it was associated with higher IC_{50}s for artesunate (ATS) (28). Whereas this substitution was considered rare in previous analyses (11, 17, 22, 55), a recent study of parasite isolates obtained from travelers to Africa suggested that it might be quite prevalent in Africa (52). However, an *in vitro* analysis of a single African isolate carrying the S769N mutation showed sensitivity to dihydroartemisinin (DHA) and ATM (11, 12). Thus, the role of the S769N mutation of PfATP6 in resistance to ARTs remains to be verified experimentally.

To elucidate a possible role of PfATP6 in ART resistance, we investigated whether the S769N mutation influenced the parasite's sensitivity to ARTs. By using a transfection technique, we replaced the wild-type *Pfatp6* allele with the S769N mutant allele by genetic recombination. Comparison of the resulting parasite

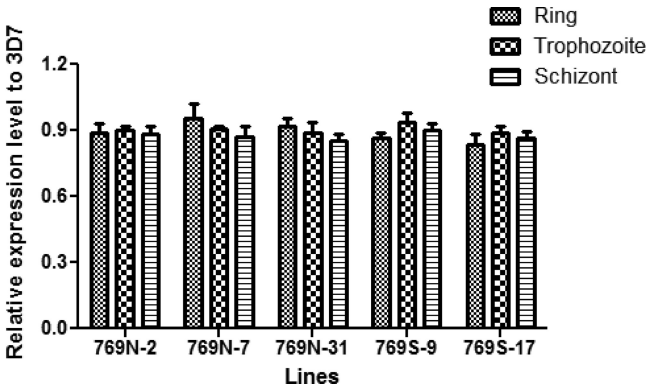

FIG 2 *Pfatp6* expression in wild-type and S769N mutant clones. *Pfatp6* expression levels are shown in the ring (12 h), trophozoite (30 h), and schizont (38 h) stages. The relative expression level of *Pfatp6* was determined by real-time PCR analysis using primers P3 and P4. A housekeeping gene, *seryl-tRNA synthetase* (PF07_0073), was used as an internal control. There were no significant differences in mRNA levels among the parasite clones at each development time point ($P > 0.05$, ANOVA).

lines failed to produce significant differences in IC_{50}s to ARTs between parasites carrying the wild-type and the mutant alleles, indicating that the S769N mutation of PfATP6 is not involved in modulating *P. falciparum* sensitivity to ARTs.

MATERIALS AND METHODS

DNA construct. The transfection construct was designed in the vector pHD22Y, which carries the human dihydrofolate reductase gene (*hdhfr*), conferring resistance to WR99210, with the *calmodulin* promoter and the *histidine-rich protein 2* terminator (23). A 2.2-kbp fragment upstream of the stop codon of *Pfatp6* was amplified using primers AGATCTCAACAC CTGTACAATCATCAAATAAG and CTCGAGTTAATCAATTTTAATT TTCTTGGTTCTTTGC (restriction sites are underlined) and cloned into a plasmid, Tg23-Luc, at the BglII and XhoI site (15). The fragment consisting of the 2,200-bp 3′ sequence of the *P. berghei dhfr-ts* gene (*PbDT-3′*) was moved into pHD22Y at the BamHI and SpeI site. Site-directed mutagenesis was performed to create the S769N mutation by using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA). Briefly, the plasmid pHD22Y-pfatp6 was amplified using two complementary oligonucleotides (5′-GCTTATAAAA AATTAAATAGTAAAGATTTAAATATTAAGAATACAGATG-3′ and 5′-CATCTGTATTCTTAATATTTAAATCTTTACTATTTAATTTTTTAT AAGC-3′), containing the desired mutation (underlined). After digestion with KpnI to remove the parental DNA template, the amplified products were used to transform bacteria, and positive clones were sequenced to confirm the presence of the S769N mutation. The plasmid containing the S769N mutation, here designated pHD22Y-pfatp6-769N, was purified for transfection.

Parasite culture and transfection. The *P. falciparum* line 3D7, with one copy of Pf*mdr1*, was cultured in human O^+ erythrocytes at 5% hematocrit in complete medium (RPMI 1640 supplemented with 25 mM HEPES [pH 7.5], 25 mM sodium bicarbonate, 50 mg/liter hypoxanthine, 0.5% Albumax II, and 40 μg/ml gentamicin sulfate) as previously described (14). Cultures were maintained at 37°C in a gas mixture of 5% CO_2, 3% O_2, and 92% N_2. Culture synchronization was performed by two rounds of treatment of ring-stage parasites with 5% (vol/vol) sorbitol for 5 min (34). Parasites were released by treatment with 0.05% saponin. Transfection of the parasite was performed using the erythrocyte loading method (18). After transfection, parasites were cultured under 2.5 nM WR99210 until resistant parasites emerged and reached 5% parasitemia. To enrich parasites with chromosomal integration of the plasmid, parasites were cultured in the absence of drug for 2 weeks. Afterwards, the parasites were cultured under 5 nM WR99210 for 2 to 3 weeks, until the parasitemia reached 5%. This drug on-off cycle was repeated three times, and resulting parasites were cloned by using the single-cell sorting method (41).

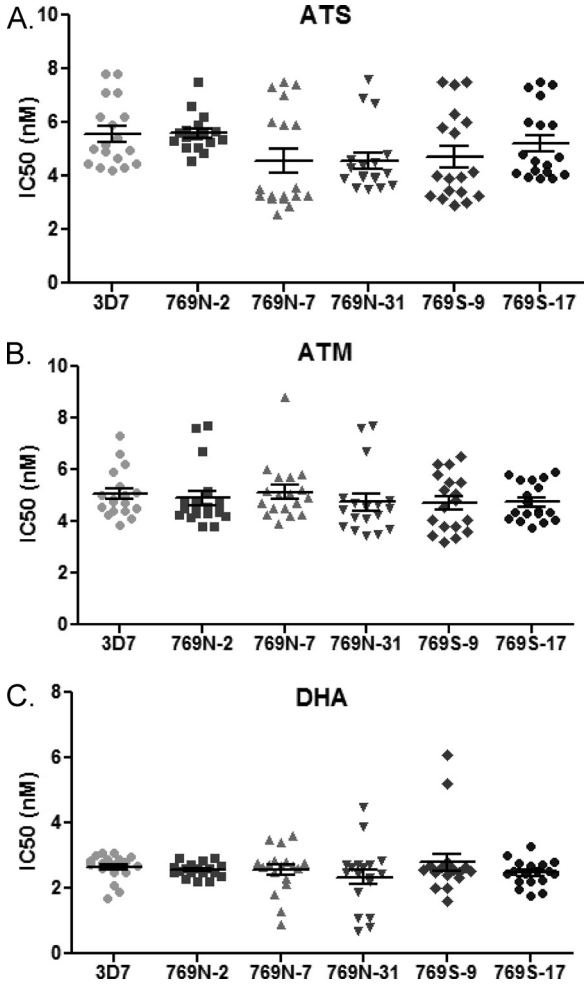

FIG 3 Scatter plot of IC_{50}s of 3D7 and five transgenic clones carrying either 769N (2, 17, and 31) or 769S (9 and 17), assayed with ATM (top), ATS (middle), and DHA (bottom). Each value indicates the mean from three technical replicates. The means ± standard deviations were calculated from the means of 18 biological experiments. For statistical comparison, data were normalized using natural logarithm transformation. For each drug, there were no significant differences among the parasite lines after controlling for multiple tests ($P > 0.05$, unpaired *t* test).

Confirmation of integration by PCR and Southern blotting. *P. falciparum* DNA was purified from saponin-released parasites using the phenol-chloroform extraction method (13). Plasmid integration at the *Pfatp6* locus was determined by integration-specific PCR using primer P1 (5′-G ATATATTACCAACATTCTC-3′), located upstream of the integration region, and P2 (5′-CATATCCGGTACCATTGTC-3′) located in *PbDT-3′* of the plasmid (Fig. 1A). PCR products from different parasites were sequenced to identify clones with the S769N mutation (Fig. 1A). To confirm the integration event at the *Pfatp6* locus, Southern hybridization was performed as previously described (42). Briefly, 3 μg of parasite DNA from each clone was digested with KpnI, separated on a 1% agarose gel, and transferred to a nylon membrane. The probe located upstream of the integration region (Fig. 1A) was amplified with primers (5′-GCTGCCGT AGGTGTATG-3′ and 5′-CCATGAATTGGATCTGAG-3′) and labeled with digoxigenin (DIG) by using a DIG PCR labeling kit (Roche Applied

TABLE 1 Unpaired *t* test results for mutant vs control lines exposed to the three drugs

Comparison	Drug	*P* value[a]
769N-2 vs 769S-17	ATS	0.0310
	ATM	0.4497
	DHA	0.1289
769N-31 vs 769S-17	ATS	0.3293
	ATM	0.8467
	DHA	0.5472
769N-7 vs 769S-9	ATS	0.0443
	ATM	0.0247
	DHA	0.7577

[a] After a Bonferonni correction, *P* values of less than 0.0056 were considered significant.

Science). The membrane was hybridized with denatured probes for 12 h at 58°C. Hybridized DNA was detected with a DIG luminescence detection kit (Roche Applied Science) and exposed to X-ray film.

To further determine whether the single-crossover events involved the integration of plasmid concatemers, the plasmid copy numbers in the integrated clones were determined by real-time PCR analysis using primers P3 (5′-GTTTTCTGTAGAACTG-3′) and P4 (5′-GATAACGGATAA ATGC-3′) (Fig. 1A). Pf*apt6* copy number was determined by comparing it with 3D7 using the $2^{\Delta\Delta CT}$ method with the single-copy gene *seryl-tRNA synthetase* (PF07_0073) as an internal reference (15).

***Pfatp6* gene expression.** To assess the expression level of *Pfatp6* in different parasite clones, total RNA was isolated from synchronized parasites at the ring (12 h), trophozoite (30 h), and schizont (38 h) stages by using TRIzol (Invitrogen, Carlsbad, CA). The RNA was directly used as template for real-time reverse transcriptase PCR (RT-PCR) analysis using a One-Step quantitative RT-PCR master mix kit (USB, Cleveland, OH). The relative expression level was calculated by comparing the result with that in 3D7 and using the $2^{\Delta\Delta CT}$ method (15). The housekeeping gene *seryl-tRNA synthetase* (PF07_0073) was used as an internal reference.

***In vitro* drug assays.** Three ART derivatives, DHA, ATM, and ATS, were purchased from Sigma (St. Louis, MO). Drug stock solutions (10 mM) were made fresh in dimethyl sulfoxide (DMSO) and stored at −80°C. *In vitro* sensitivities of the parasite lines to ART derivatives were determined by using the SYBR green I method with 2-fold serial dilutions of the drugs to final concentrations of 0.8 to 200 nM (37, 54). Briefly, 100 μl of a ring-stage parasite culture at 0.5% parasitemia and 1% hematocrit in the culture medium with different drug concentrations was seeded in triplicate in 96-well flat-bottom plates and incubated at 37°C for 72 h. Afterwards, the plates were frozen and thawed, mixed with 100 μl of lysis buffer, and incubated in the dark at 37°C for 4 h. Fluorescence was measured using the FLUOstar OPTIMA microplate reader (BMA Labtech, Offenburg, Germany) with excitation and emission wavelengths centered at 485 and 538 nm, respectively. For accuracy, each parasite line was measured in 18 biological replicates, each with three technical replicates. IC_{50}s were calculated using the program GraphPad Prism version 5 (La Jolla, CA) by constructing a dose-response curve. The percentage of inhibition was calculated using the following formula: [(fluorescence of drug treated parasites − fluorescence of untreated control)/(fluorescence of untreated control)] ×100.

Statistical analysis. The mean IC_{50}s of mutant parasite clones were compared to the mean IC_{50}s of control lines by using unpaired *t* tests, assuming unequal variances. The data were first transformed using the natural logarithm in order to control for nonnormality in IC_{50}s. In order to control for multiple tests, both a Bonferroni correction and a Benjamini-Hochberg correction were applied to the *t* test results.

RESULTS

Development of transgenic lines expressing the PfATP6 S769N mutation. To determine whether PfATP6 with the S769N mutation is able to modulate susceptibility of *P. falciparum* to ARTs, we generated transgenic parasites lines expressing the S769N mutant PfATP6 in 3D7 by using a single-crossover strategy (Fig. 1A). After transfection of the 3D7 parasite with the pHD22Y-pfatp6-769N construct, parasites were selected with WR99210 until resistant parasites appeared in 3 weeks. Afterwards, parasites were cultured through three drug on-off cycles to enrich parasites with chromosomal integration of the plasmid. To obtain multiple mutant clones with the S769N mutation, 200 clones were obtained by single cell sorting and analyzed. Integration-specific PCR using primers P1 and P2 (Fig. 1A) identified 32 positive clones with the correct integration event occurring at the *Pfatp6* locus (Fig. 1B). After sequencing each PCR product of the 32 clones, three clones (769N-2, 769N-7, and 769N-31) were found to harbor the S769N mutation (Fig. 1B). Since genetic recombination with single crossover often results in the insertion of plasmid concatemers, we

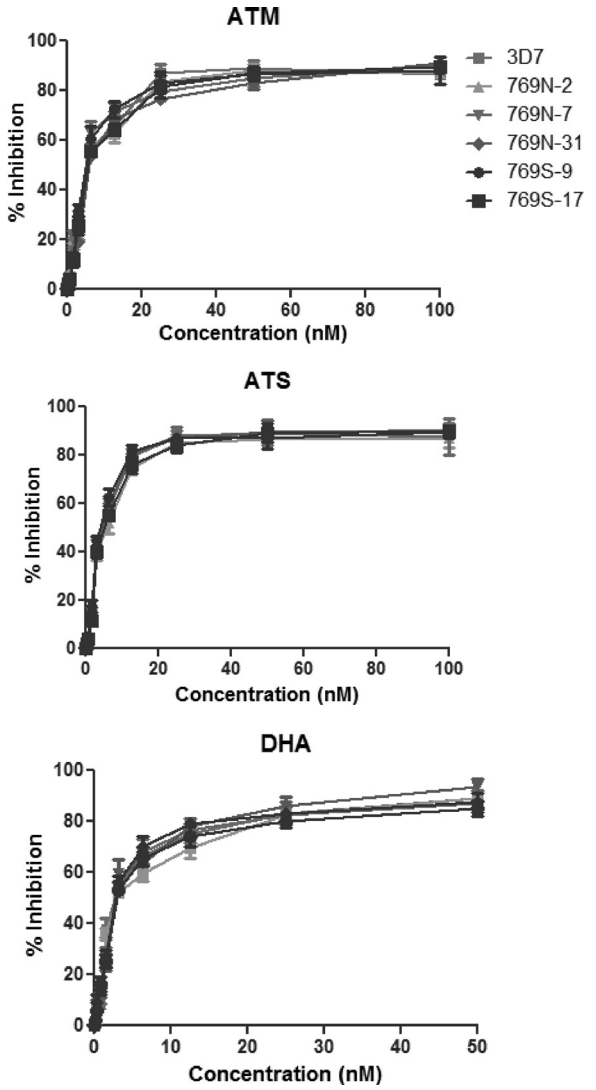

FIG 4 Dose-response curves of 3D7 and five parasite clones with either 769N (2, 17, and 31) or 769S (9 and 17) assayed against ATM (top), ATS (middle), and DHA (bottom). The results were obtained from 18 independent experiments, each with three technical replicates. Percent inhibition values are shown as means ± standard deviations.

determined the copy number of the integrated plasmid in all 34 clones by real-time PCR analysis. The result showed that clones 769N-2 and 769N-31 had one copy of the plasmid inserted into the genome, whereas 769N-7 had three copies of the plasmid. To eliminate the possible effects of insertion of different copies of the plasmid on sensitivities to ARTs, two parasite clones (769S-17 and 769S-9) from similar integration events but without the S769N mutation were chosen as transfection controls. Clones 769S-17 and 769S-9 had one and three copies of the plasmid integrated, respectively (Fig. 1C). The integration events of the selected five clones were further confirmed by Southern blotting (Fig. 1D). As predicted, a 10-kb KpnI fragment was detected in 3D7 parasite genomic DNA, whereas a 5.8-kb KpnI fragment was observed after the integration of the plasmid at the *Pfatp6* locus (Fig. 1D).

***Pfatp6* gene expression.** To determine whether the integration events affected *Pfatp6* expression, the mRNA levels of *Pfatp6* in the five selected clones were compared using real-time RT-PCR with 3D7 as the reference. No significant difference in *Pfatp6* expression was detected among the five clones ($P > 0.05$, ANOVA) (Fig. 2A). This result was consistent with the prediction, since for clones with insertion of more than one copy of the plasmid, only the first copy of the *Pfatp6* gene had a promoter and was transcribed.

***In vitro* response of transgenic lines to ART derivatives.** We next determined the IC_{50}s of 3D7, three mutant lines (769N-2, -7, and -31) and two control lines (769S-9 and -17) to the three ART derivatives. The IC_{50} of each parasite clone to each of the ART derivatives was determined in 18 biological replicates, each with three technical replications (Fig. 3). All transfectant lines and 3D7 had similar IC_{50}s against ATM, ATS, and DHA. The means of absolute IC_{50}s of the five transgenic lines were log transformed and compared for statistical significance. Statistical analysis confirmed the lack of a significant difference in IC_{50}s between mutant lines and their control lines (Table 1) ($P > 0.0056$, unpaired *t* tests). We further compared the dose-response curves of all parasite lines and found that the dose-response patterns for the ART derivatives were very similar (Fig. 4).

DISCUSSION

The mode of action of ARTs in malaria parasites is still not completely understood, and the molecular basis of reduced ART susceptibility is unclear (16, 33, 38). So far, a number of genes have been proposed to be associated with reduced sensitivities to ARTs, but none of the associations has been conclusively validated (19). Based on heterologous expression studies and biochemical assays, PfATP6 has been postulated to be a prime target of ART, and L263E was considered a potential mutation that mediatesART resistance (21, 56). These initial studies spurred extensive investigations on PfATP6 (31), but results obtained so far have cast considerable doubts on the role of PfATP6 in ART resistance. Biochemical studies of purified PfATP6 failed to detect inhibition of this enzyme by ARTs (2, 7), suggesting that findings from heterologous expression in *Xenopus* oocytes may be a system-specific effect. In this study, we further evaluated the potential role of PfATP6 in ART resistance, and our allele exchange experiments confirmed the lack of association of the S769N mutation of this gene with altered sensitivity to ARTs.

Two mutations in PfATP6 at positions 263 and 769 have been linked to ART resistance. The L263E mutation was proposed based on docking simulation and found to confer insensitivity to ARTs in the *Xenopus* oocyte system (56). The L263E mutation has so far eluded detection in field parasite populations, and introduction of L263E in *P. falciparum* through allele exchange did not cause significant changes of parasite sensitivities to ARTs (57). While such a discrepancy is not clearly understood, a recent docking simulation study suggested that the significance of L263E in ART resistance may be less than previously hypothesized (35). The S769N mutation was originally found in *P. falciparum* field isolates from French Guiana, and it was linked to increased resistance to ATM (27). Although the S769N mutation has been found in some parasite isolates from Africa (11, 28, 52), a drug assay on a single parasite isolate showed that it was sensitive to DHA and ATM (11, 12). In this study, we showed that introduction of the S769N mutation in 3D7 by an allele exchange strategy did not alter the parasite's sensitivity to all tested ART derivatives. Whereas these data argue against the predicted role of these PfATP6 mutations in modulating ART sensitivity, it remains to be determined whether the divergent findings are due to different genetic backgrounds of the parasite lines. It has been reported that genetic backgrounds of the parasites may greatly influence the effect of *Pfcrt* on CQ resistance (57), *Pfmdr1* on resistance to CQ and quinine (50, 53), and *Pfnhe1* on quinine resistance (6, 37).

Sequencing of *PfATP6* has identified many mutations in this gene among field parasite populations (25), but none of them have been conclusively linked to ART resistance. Some studies showed that deployments of ACTs were associated with changes of frequencies of certain mutations in PfATP6. In one study the frequency of the A623E mutation was increased in Niger after ACT use (3), whereas in another study an increase in the frequency of a deletion mutant was noticed in Peru (8, 49, 64). In the GMS, an epicenter of malaria drug resistance with the most extensive use of ART drugs, A623E and S769N mutations associated with reduced sensitivity to ARTs have not been detected so far (26). In addition, the clinical ART resistance in western Cambodia is not associated with *PfATP6* (55). It is noteworthy that most of the PfATP6 mutations are rare and geographically confined. Molecular evolution analysis of PfATP6 single-nucleotide polymorphisms showed that the ratio of synonymous versus nonsynonymous substitutions did not significantly deviate from neutrality (59a). Moreover, our analysis of parasite samples collected from the GMS after deployment of ARTs revealed similar findings (37). Collectively, the evidence accumulated thus far strongly suggests that PfATP6 does not have much to do with ART resistance.

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