MAJOR ARTICLE

Expression in Yeast Links Field Polymorphisms in PfATP6 to in Vitro Artemisinin Resistance and Identifies New Inhibitor Classes

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Background. The mechanism of action of artemisinins against malaria is unclear, despite their widespread use in combination therapies and the emergence of resistance.

Results. Here, we report expression of PfATP6 (a SERCA pump) in yeast and demonstrate its inhibition by artemisinins. Mutations in PfATP6 identified in field isolates (such as S769N) and in laboratory clones (such as L263E) decrease susceptibility to artemisinins, whereas they increase susceptibility to unrelated inhibitors such as cyclopiazonic acid. As predicted from the yeast model, *Plasmodium falciparum* with the L263E mutation is also more susceptible to cyclopiazonic acid. An inability to knockout parasite SERCA pumps provides genetic evidence that they are essential in asexual stages of development. Thaperoxides are a new class of potent antimalarial designed to act by inhibiting PfATP6. Results in yeast confirm this inhibition.

Conclusions. The identification of inhibitors effective against mutated PfATP6 suggests ways in which artemisinin resistance may be overcome.

Keywords. Artemisinins; PfATP6; yeast; malaria; *Plasmodium falciparum*; drug resistance; thaperoxides; cyclopiazonic acid; desferioxamine.

Schatzmann's observation that cardiac glycosides inhibited active exchange of Na^+ and K^+ across red cell membranes was critical to the discovery of Na^+/K^+ ATPase as their target [1]. Subsequently, activated benzimidazoles were found to inhibit parietal cell H^+/K^+ ATPases [2], thereby providing effective treatments for inappropriately elevated gastric acid. These studies established the value of P-type ATPases as targets for distinct chemical classes used to treat a variety of unrelated diseases. Artemisinins are key components of most commonly used antimalarial combination therapies. They were hypothesized to act by inhibiting a parasite-encoded P-Type Ca^{2+} ATPase (PfATP6) of the SERCA family [3]. Consistent with this suggestion, PfATP6 was inhibited after expression in *Xenopus laevis* oocytes. Mutations in PfATP6 (eg, L263E) were predicted and then found to decrease sensitivity to artemisinins in this assay [4], in which approximately 1% of membrane-associated protein may be PfATP6 [5]. Amino acid polymorphisms at other positions in PfATP6 were subsequently associated with decreased susceptibility of field parasites to artemisinins (reviewed in [6–8]). However, introducing mutations in PfATP6 into cloned parasite lines generates phenotypes that vary in sensitivity to artemisinins [9, 10]. Also, highly purified PfATP6 reconstituted in artificial membranes is not inhibited by artemisinins and only poorly by thapsigargin, a specific SERCA inhibitor [11] that is predicted in modeling studies to inhibit PfATP6 [12]. To resolve

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these discrepancies, we developed a whole cell yeast expression system to study PfATP6. This model conveniently allows assessments of how polymorphisms in PfATP6 in field isolates relate to the emerging problem of artemisinin resistance. It also clarifies mechanisms of antiparasitic action of this class of drug.

Results associate polymorphisms in PfATP6 linked to artemisinin resistance in vitro in French Guiana and Sénégal [13] with decreased sensitivity to artemisinins of yeast-expressed PfATP6 sequences. They also independently validate PfATP6 as a drug target by genetic experiments and identify new inhibitors as leads for antimalarials.

RESULTS

Genetic Studies of PfATP6

Genetic validation of PfATP6 as a drug target has not been reported to our knowledge. We carried out knockout (KO)/complementation experiments in the reference line 3D7 with a single crossover homologous recombination strategy (Figure 1*A*), as with the glucose transporter, PfHT [14]. Two attempts were unsuccessful, including complementation with wild-type or double mutated full length *pfatp6* (Figure 1*C*) and tagging the 3′ end of the gene with green fluorescent protein (GFP), *myc,* or double HA epitopes (Figure 1*B*). In all cases, blasticidin-resistant parasites were obtained with episomes. Similarly, a double recombination construct failed to KO *pbserca* in the more tractable *Plasmodium berghei* system, and C-terminal tagging was also unsuccessful (data not shown). Thus, *pfatp6* is accessible for crossovers introducing single mutations, supporting the idea that it cannot be knocked out [9, 10] or tagged at the C-terminus. We developed an alternative expression system to study the function of PfATP6 and mutations including those in field isolates.

Yeast Expression

To avoid extensive purification and reconstitution after expression in yeast [11] or laborious assays of membrane preparations of *Xenopus* oocytes [3], we adapted a yeast functional assay used to express several exogenous eukaryotic Ca^{2+} ATPases [15]. This assay has the advantage of assessing drug action in intact cells. The host strain (K667) lacks one of its 2 Ca^{2+} ATPase genes *PMC1*, whereas the other gene, *PMR1*, is inactive as calcineurin is also missing. A codon-optimized version of *pfatp6* was required for yeast expression [11].

PfATP6 was localized to cytosolic structures in yeast (Figure 2*A*, *upper panel*) with a similar pattern in parasites (Figure 2*A*, *lower panel*) that is consistent with distribution of endoplasmic reticulum (ER). To verify this localization, PfATP6 was compared in ER-enriched microsomal membranes and

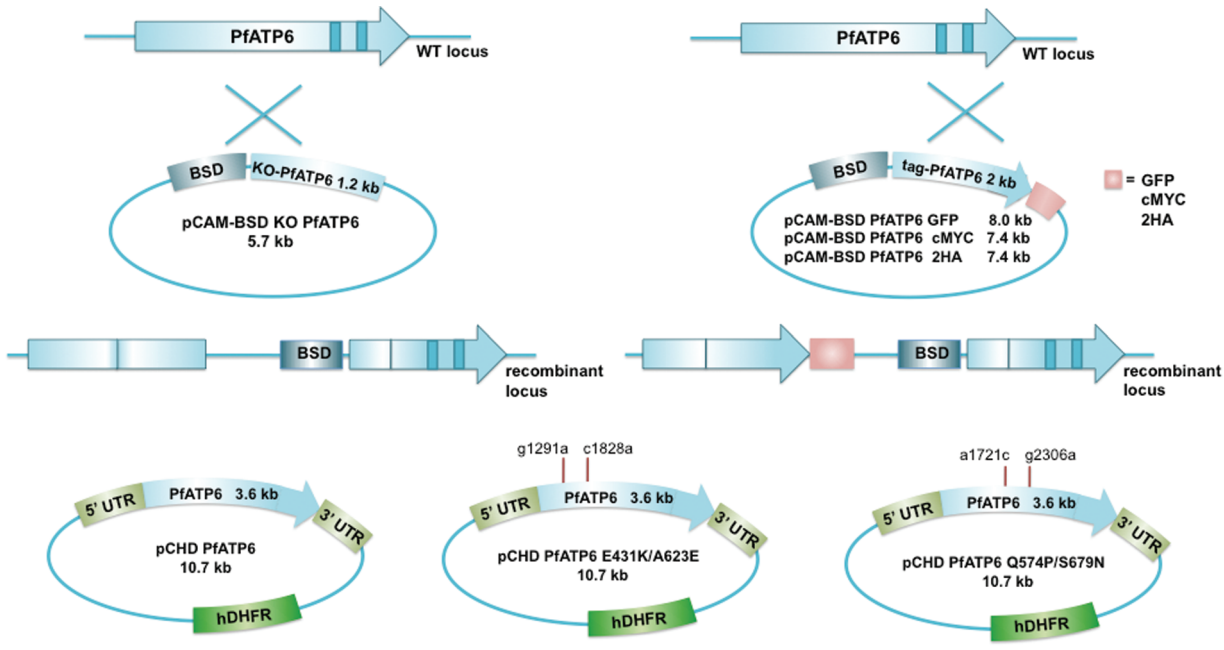

Figure 1. Strategy for knockout (KO), complementation and tagging of *pfatp6* gene in *Plasmodium falciparum* 3D7. *A*, Single cross-over homologous recombination of the KO (pCAM-BSD KOA6) plasmid and the endogenous gene (WT locus) should result in 2 truncated nonfunctional copies of the gene (recombinant locus). *B*, Tagging of the 3′ end of PfATP6 locus with green fluorescent protein (GFP), double HA or c*myc* tags. Wild-type (WT) and recombinant loci are shown. Arrows indicate the location of polymerase chain reaction (PCR) primers. *C*, Complementation constructs, carrying WT and mutated PfATP6 (pCHD-A6) would allow PfATP6 expression under the Pfhsp86 promoter. Complementation of KO parasites without a tagged *pfatp6* construct was not successful, and neither was episomal expression only of PfATP6.

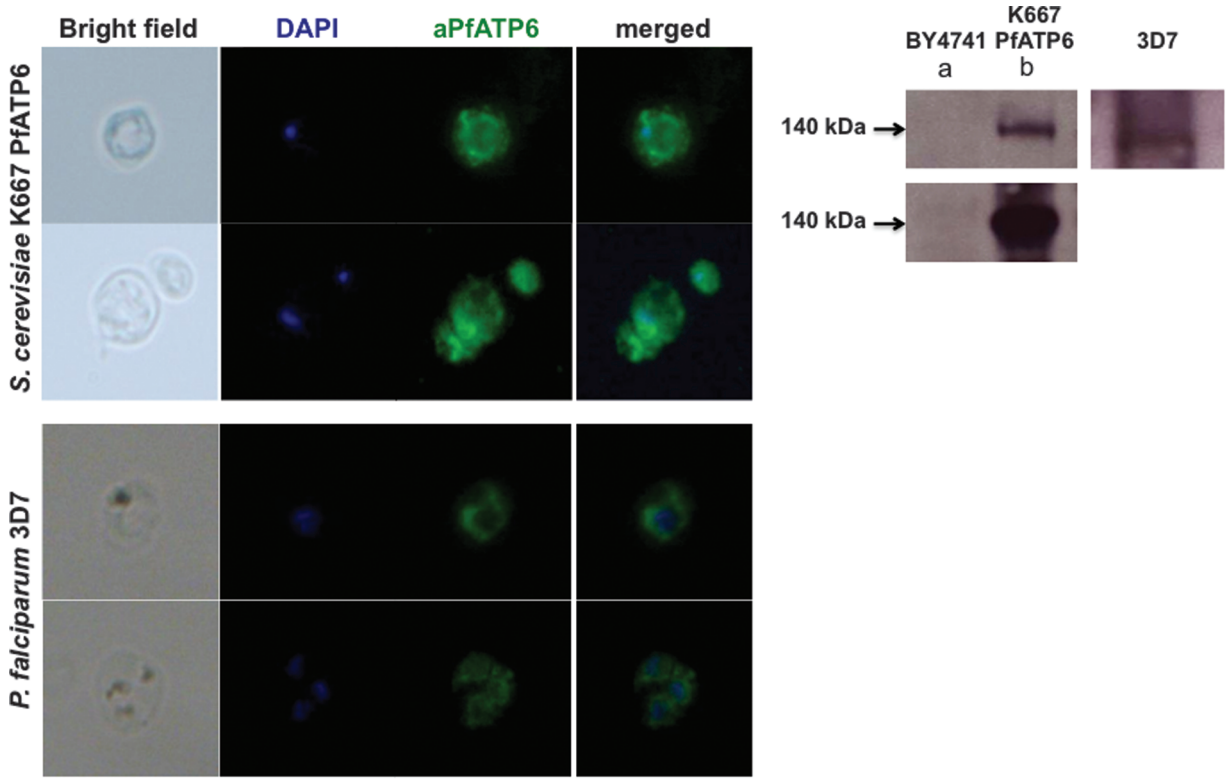

Figure 2. PfATP6 in yeast and parasites. *A, Upper panel*: Indirect immunofluorescence with anti-PfATP6 polyclonal antibody on *Saccharomyces cerevisiae* K667::*pfatp6* confirms expression in cytosolic structures (*green*) with nuclei stained with DAPI (*blue*). *Lower panel*: A similar distribution is apparent in asexual stage parasites (large trophozoites of *Plasmodium falciparum* strain 3D7). *B, Upper panel*: Western blot of total protein extract of yeast (10 µg, *left*) and parasites (10 µg, *right*). Reference strain BY4741 (lane *a*) is a negative control, whereas K667::*pfatp6* shows a band of predicted mass (*upper arrow*). A similar band is detected on parasite extracts (3D7). *Lower panel*: Reference yeast strain (BY4741, 10 µg lane *a*) and K667::*pfatp6* were used to make ER-enriched preparations (Methods) and assayed with anti-PfATP6 antibody. Only K667::*pfatp6* (10 µg lane *b*) shows PfATP6.

whole cell extracts from yeast by Western analysis (Figure 2*B*). Endogenous yeast non-SERCA P type Ca^{2+} ATPases (Pmr1p and Pmc1p) in reference strain BY4741 are not detected by anti-PfATP6 anti-peptide polyclonal reagent (Figure 2*B*, *lane a*). PfATP6 is detected in membrane extracts from both transgenic yeast and parasites (*P. falciparum* 3D7; Figure 2*B*, *lane b and right panel*).

Yeast Assays

To confirm function, we first established conditions for yeast growth that depend on rescue by PfATP6. A reference strain (BY4741) with 2 Ca^{2+} ATPases can grow on solid (YPD) medium supplemented with $CaCl_2$ (0–100 mM, Figure 3*A*, *upper row*). As expected, strain K667 cannot grow when stressed with ambient calcium concentrations >25 mM (Figure 3*A*, *2nd row*), and the addition of an empty vector (K667::pUGpd; Figure 3*A*, *3rd row*) does not alter this calcium sensitivity. Growth of K667 is restored toward that of the reference strain by expressing PfATP6, albeit not to wild-type levels (K667::*pfatp6*; Figure 3*A*, *last row*).

Liquid cultures allow quantitation of growth and show that PfATP6 expressing yeast (OD_{620} nm) grow significantly better in media supplemented with ≥50 mM $CaCl_2$ compared with yeast strains (K667 and K667::pUGpd) without Ca^{2+} ATPases (Figure 3*B*; $P < .001$). PfATP6 cannot restore growth in increasing manganese concentrations, confirming that (as with reconstituted protein [11]) it acts specifically as a Ca^{2+} ATPase. This manganese sensitivity also confirms that the Ca^{2+}/Mn^{2+} ATPase PMR1 is inactive in K667 (Figure 3*C*). Growth curves for yeast in different calcium concentrations were used to select 42 hours as the time point for assessing effects of drugs (Supplementary Figure 1). This allows discrimination of growth rates at the plateau phase (see Methods). The mean ± standard deviation (SD) interassay coefficient of variation (CV) for pUGpd was 1.85% ± 0.017% (calculated as an overall mean (n = 28) using the mean CV of 3 biological replicates after assays with artemisinins and cyclopiazonic acid [CPA] at all reported concentrations) and for K667::*pfatp6* was 6.65% ± 0.07% (calculated as for pUGpd with 40 mM $CaCl_2$ in medium, and including assays for chloroquine and thapsigargin (n = 36)).

BY4741 growth without calcium is inhibited by artemisinins perhaps by inhibition of yeast mitochondrial function [16] and through effects on its own Ca^{2+} ATPases [17]. Strain K667 can

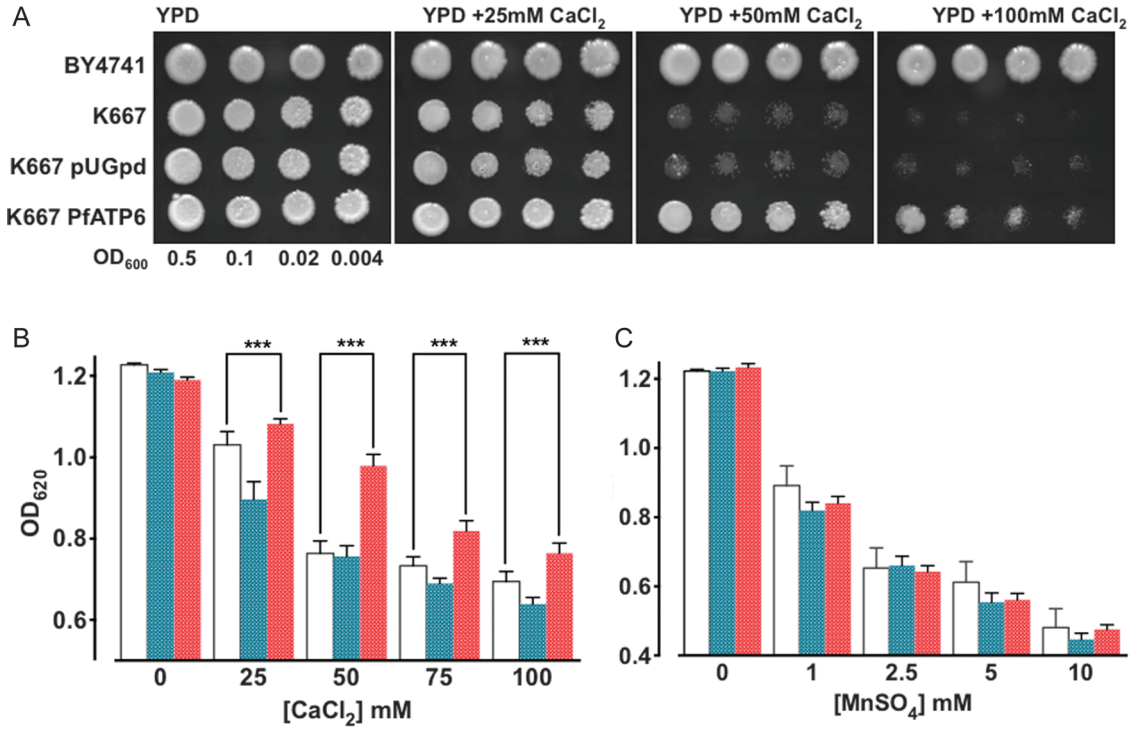

Figure 3. Functional assay of PfATP6 in growing yeast. *A*, Saturated liquid cultures of yeast cells were diluted (as indicated by OD_{600} values) and spotted onto yeast peptone dextrose (YPD) plates supplemented with 25, 50, or 100 mM $CaCl_2$. BY4741 (*upper row*) tolerates all calcium concentrations whereas K667 and K667::pUGpd (bearing empty vector; *middle rows*) cannot grow in calcium ≥50 mM. PfATP6 (K667::*pfatp6*) restores growth in higher ambient calcium (*lower row*). *B*, K667 (*bars*), K667::pUGpd (*red bars*) and K667::*pfatp6* (*blue bars*) were diluted to a cell density of 0.5 and inoculated into YPD medium containing $CaCl_2$ at indicated concentrations. Yeast cell density was determined after growth at 30°C for 18 h. Data are mean ± SEM values of at least 9 replicates (3 independent biological experiments). PfATP6 rescue increases growth of K667 significantly (***$P < .0001$). *C*, K667 (*bars*), K667::pUGpd (*blue bars*), and K667::*pfatp6* (*red bars*) were diluted to a cell density of 0.5 and inoculated into YPD medium containing $MnSO_4$ at indicated concentrations. Data are mean ± SEM values of at least 9 replicates (3 independent biological experiments). There are no significant differences between groups ($P > .05$).

only be grown without calcium, so artemisinins can still inhibit growth by acting on mitochondria but not on yeast Ca^{2+} ATPases. We therefore used 40 mM $CaCl_2$, in liquid cultures to screen artemisinins for their ability to inhibit rescue of K667 by PfATP6. These conditions do not permit K667 to grow without PfATP6, allowing the conclusion that inhibition is of PfATP6's ability to rescue yeast. All artemisinins inhibited yeast growth significantly (Figure 4*A*, $P < .001$).

CPA inhibits SERCAs at a different binding site from thapsigargin [18] (where we hypothesized that artemisinins may act), and significant inhibition is also observed with CPA (Figure 4*A*). Several experiments confirmed the specificity of these inhibitors. Growth of K667 is not inhibited by dimethyl sulfoxide solvent (1% maximum, not shown) and CPA (up to 100 μM, Figure 4*B*) did not inhibit any strain without PfATP6 (BY4741, K667, K667::pUGpd).

Table 1*A* includes results with different concentrations of inhibitors of K667::*pfatp6*. 2-deoxy artemisinin lacks an endoperoxide bridge and is therefore inactive as an antimalarial and interestingly does not inhibit growth. Chloroquine, an antimalarial that acts in the parasite's food vacuole, also does not inhibit K667::*pfatp6*. As is conventional, we are careful not to translate concentrations that inhibit K667::*pfatp6* to those required to kill parasites. Higher concentrations of ATPase inhibitors are needed to inhibit yeast growth compared with potencies in biochemical assays [18].

Results from K667::pUGpd (vector control) are not directly comparable to those with K667::*pfatp6*, as the former have been grown without supplementary calcium. Supplementary Table 1*A* presents the effects of inhibitors on pUGpd, representing inhibition that is not dependent on PfATP6. Most inhibitors have little or no effect. Thapsigargin and CPA inhibit growth in K667::*pfatp6* but not in K667::pUGpd, consistent with a specific interaction with PfATP6 (Table 1*A* and Supplementary Table 1*A*). Some artemisinins inhibit K667::pUGpd at higher concentrations (for example, artemisone (100 μM) inhibits by approximately 25%) but all Ca^{2+} ATPase inhibitors (including artemisinins) inhibit K667::*pfatp6* significantly more.

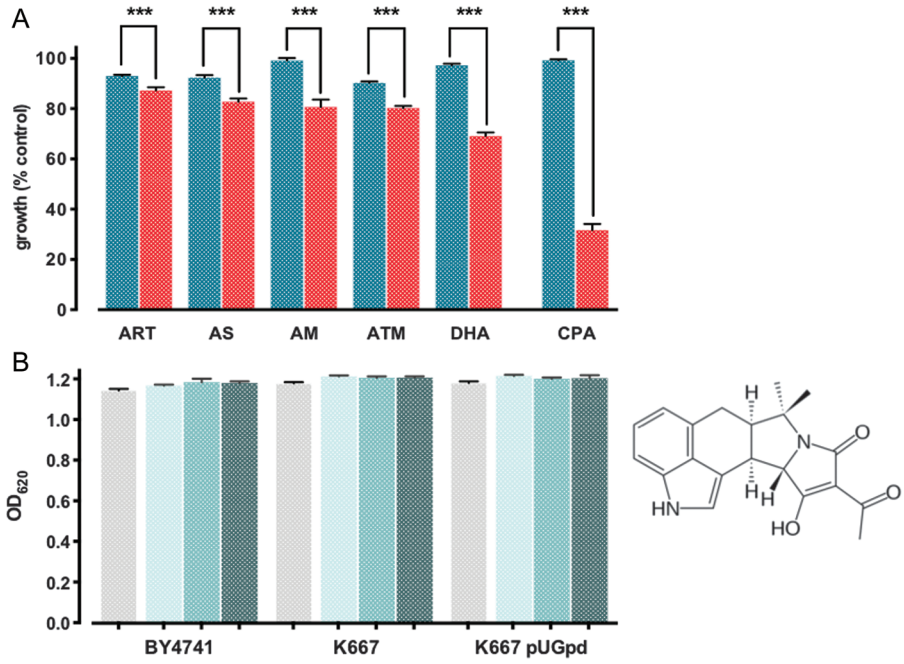

Figure 4. Sensitivity of K667 with and without rescue by PfATP6 to artemisinins and cyclopiazonic acid. *A*, Inhibition of K667::pUGpd (*blue bars*) and K667::*pfatp6* (*red bars*) by different antimalarials expressed as percentage of control cultures (growth of each strain in absence of the drug). Yeast strains were diluted to a cell density of 0.1 and inoculated into YPD medium (with 40 mM $CaCl_2$ for experiments with K667::*pfatp6*) and 10 μM of drug as indicated. Yeast cell density was determined after growth at 30°C for 42 h (plateau phase, Supplementary Figure 4). Data are mean ± SEM values of at least 9 replicates (3 independent biological experiments) with significant differences between growth of yeast without PfATP6 and supplementary Ca^{2+} and yeast with PfATP6 and supplementary Ca^{2+} (***$P < .001$). *B,* CPA does not inhibit growth of yeast strains not expressing PfATP6 (BY4741, K667 and K667::pUGpd). Yeast strains were diluted to a cell density of 0.1 and inoculated into YPD medium containing various concentration of drug as indicated (*bars*, no drug, *light green*, 1 μM, *medium green*, 10 μM, *dark green*, 100 μM). CPA structure is reported. Abbreviations: AM, artemether; ART, artemisinin; AS, artesunate; ATS, artemisone; CPA, cyclopiazonic acid; DHA, dihydroartemisinin.

To confirm that mitochondrial inhibition by artemisinins is not relevant to inhibition of K667::*pfatp6*, we assessed the effects of adding deferoxamine (DFO). Previously, the artemisinin susceptibility of yeast mitochondria was antagonized by DFO [16]. In contrast, DFO increased the inhibition of yeast growth by artemisinin (10 μM, Supplementary Table 1*B*) and had no effect on actions of artemether or artemisone. At the highest concentrations of artesunate there was some antagonism with DFO.

Mutations in PfATP6

Next we tested how mutations in PfATP6 influence artemisinin activity. These assays (in extracellular calcium between 10 and 30 mM) examined mutations (L263E, A623E, S769N A623E/S769N) by comparing results with wild-type PfATP6 and vector only controls (Figure 5*A*, *lower panel*).

All *pfatp6* constructs rescued K667 growth in 10- and 20-mM calcium, although S769N and A623E/S769N mutants were significantly less efficient in 20-mM calcium. In 30-mM calcium, all mutants of PfATP6 failed to rescue yeast in contrast to wild-type PfATP6 (Figure 5*A*, *lower panel*). Western analysis confirmed expression of mutant PfATP6 proteins (Figure 5*A*, *upper panel*). Mutations in PfATP6 reduce the efficiency of yeast rescue, presumably by interfering with function. We then tested sensitivity of mutant PfATP6 sequences to artemisinins and compared them with wild-type PfATP6 (Table 1*B* and Supplementary Table 2 for 95% confidence intervals [CIs] of mean differences between groups). S769N attenuated artemisinins' inhibition (except for artesunate 1 μM and artemisone 100 μM). For a double mutant (PfATP6 A623E/S769N) there was also significant attenuation of inhibition by all artemisinins (10 μM). In contrast, higher concentrations of artemisone (100 μM) [19] produced even greater inhibition on all mutants. The A623E mutant had less marked effects, failing to attenuate artesunate's inhibition.

In *Xenopus* oocyte membrane preparations, there was abolition of sensitivity of PfATP6 to artemisinins [4]. We confirmed in yeast that L263E PfATP6 significantly reduces but does not abolish sensitivity to all artemisinins (Table 1*B*), perhaps reflecting differences in 3 amino acid residues in PfATP6 sequence expressed in *Xenopus* compared with the wild-type (3D7) PfATP6 sequence expressed in yeast (sequence differences

Table 1. Sensitivity of K667 Expressing Wild Type and Mutated PfATP6 to Antimalarials

(μM)	Growth (% control) PfATP6
(*A*) artemisinin	
1	100.2 ± 1.1
10	87.3 ± 1.2*
100	76.4 ± 1.2*
artesunate	
1	90.1 ± 0.8
10	82.8 ± 1.3*
100	72.6 ± 0.8*
artemether	
1	94.1 ± 2.7
10	80.7 ± 3.0**
100	77.1 ± 2.8**
artemisone	
1	90.0 ± 0.3*
10	80.2 ± 0.9*
100	56.3 ± 1.2*
DHA	
1	80.9 ± 0.9*
10	69.1 ± 1.4*
100	65.8 ± 3.0*
CPA	
1	79.5 ± 6.7**
10	31.7 ± 2.4**
100	24.0 ± 1.7**
thapsigargin	
1	100.5 ± 0.5
10	99.5 ± 0.6
100	86.9 ± 0.8*
chloroquine	
1	101.6 ± 1.4
10	102.3 ± 1.0
100	100.4 ± 1.2
2de-QHS	
1	100.9 ± 1.4
10	103.0 ± 1.5
100	98.1 ± 0.9

(μM)	PfATP6 wt	A623E/S769N	A623E	S769N	L263E
(*B*) artemisinin					
1	101.9 ± 1.0	104.7 ± 1.2	104.3 ± 1.7	105.3 ± 1.7	104.4 ± 2.1
10	91.7 ± 0.6	101.7 ± 1.1***	100.5 ± 1.6***	105.3 ± 1.8***	104.5 ± 1.9***
100	71.8 ± 2.9	91.8 ± 0.5***	73.6 ± 1.8	82.9 ± 0.5****	75.4 ± 2.2
artesunate					
1	96.6 ± 0.6	97.9 ± 2.0	96.6 ± 0.9	91.9 ± 1.1####	98.8 ± 0.9
10	85.1 ± 0.9	91.1 ± 1.1***	86.9 ± 0.6	87.8 ± 1.1	89.0 ± 0.6****
100	70.5 ± 2.3	85.4 ± 0.9***	70.2 ± 2.4	78.5 ± 0.8****	72.2 ± 2.2
artemether					
1	100.1 ± 0.6	104.1 ± 1.5*****	100.0 ± 1.1	100.9 ± 1.5	103.5 ± 1.4*****
10	87.9 ± 1.5	95.7 ± 1.8****	99.7 ± 1.6***	103.0 ± 1.2***	104.2 ± 1.3***
100	65.7 ± 2.4	59.0 ± 4.1	64.6 ± 1.4	74.8 ± 1.6****	69.3 ± 2.4

Table 1 continued.

(μM)	Growth (% control) PfATP6				
artemisone					
1	91.9 ± 0.7	100.3 ± 0.6***	98.4 ± 1.7****	96.4 ± 1.8*****	101.0 ± 2.0***
10	83.1 ± 0.7	96.5 ± 1.8***	86.9 ± 1.2*****	91.9 ± 1.3***	89.6 ± 1.7****
100	45.2 ± 4.2	56.1 ± 1.9	34.5 ± 2.2#####	30.9 ± 1.7####	34.0 ± 2.8#####
DHA					
1	90.7 ± 0.7	92.0 ± 1.7	94.9 ± 0.9****	91.8 ± 2.1	95.8 ± 1.1****
10	82.7 ± 0.6	88.5 ± 1.7****	82.2 ± 1.1	82.1 ± 2.3	80.1 ± 1.1
100	61.7 ± 3.0	72.6 ± 2.7*****	63.9 ± 3.1	60.7 ± 6.5	62.8 ± 3.4

(*A*) Sensitivity of K667 expressing PfATP6 to antimalarials. Results (mean±SEM) are expressed as percentage of control (growth in absence of drug). Data are the mean of at least 9 replicates in 3 independent experiments. Growth in presence of drug was compared to growth without drug (in 40 mM $CaCl_2$) with ANOVA. (*B*) Sensitivity of K667 expressing PfATP6 (wild-type or mutated, as shown) to artemisinin derivatives. Growth is described as percentage of control (growth in YPD medium supplemented with 20 mM $CaCl_2$) Data are mean ± SEM values of at least 9 replicates (3 independent experiments). Student *t* test was used to compare growth of mutated PfATP6 to wild-type. (Supplementary Table 2 summarizes 95% CAs of mean difference.) *indicates decreased sensitivity when compared to PfATP6 wild-type, whereas # indicates increased sensitivity.

Abbreviations: CPA, cyclopiazonic acid; DHA, dihydroartemisinin.

* $P < .0001$.

** $P < .0005$.

*** $P < .0009$.

**** $P < .0061$.

***** $P < .0385$.

between PfATP6 in oocytes and the 3D7 sequence can be compared with accession numbers PF3D7_0106300 and the original sequence in [3]). Results (Table 1*B*) suggest that >1 mutation in PfATP6 may synergize to reduce sensitivity to artemisinins.

It is striking that despite reduced capacity of mutant PfATP6 sequences to rescue yeast grown in calcium, they show decreased rather than increased sensitivity to artemisinins. To confirm this effect is specific for artemisinins, we tested sensitivity of mutants to CPA and found it was increased (Figure 5*B*) compared with wild-type PfATP6. We then examined parasites with the L263E mutation to determine if they had increased sensitivity to CPA, as predicted by results in yeast. Figure 5*C* demonstrates that the IC_{50} for CPA approximately halved, compared with an isogenic control (mean ± SEM for L263 = 10.3 ± 2.7 compared with 263E = 4.7 ± 1.1 μM; n = 5 $P = .011$). There was no change in sensitivity to chloroquine (0.12 ± 0.01 compared with 0.14 ± 0.01 μM respectively).

New Chemical Classes

Figure 6*A* shows how a new chemical class introducing an endoperoxide bridge into the sesquiterpene scaffold of thapsigargin (guaianolide-endoperoxides [12]) inhibits K667::*pfatp6* without inhibiting control yeast strains (eg, growth of strain BY4741 with or without calcium supplementation (Figure 6*B*)).

DISCUSSION

Whole cell expression in yeast provides new insights into PfATP6 as a drug target as well as a screening tool for discovery of inhibitors. Results confirm findings from *Xenopus* oocytes that all endoperoxides tested inhibit PfATP6 [3]. Yeast assays have also elucidated functions of a *Schistosoma mansoni* Ca^{2+} ATPase [20], a *Fasciola hepatica* plasma membrane Ca^{2+} ATPase and mammalian SERCA1a. SERCA1a is sensitive to thapsigargin [18] as well as to artemisinin [21], suggesting that experiments with purified and relipidated PfATP6 may compromise assessment of different inhibitor classes [11, 22]. Reconstitution may not achieve full physiological functionality as PfATP6 operates at one third the rate of mammalian SERCA [11].

Confounding by off-target effects of artemisinins in this yeast model [16] has been minimized because assays have been designed to rely on function of PfATP6, by selecting fermentative conditions when mitochondria are metabolically inactive. Also, the lack of antagonism (and sometimes synergism) by DFO of artemisinin inhibition of PfATP6 contrasts with antagonism in yeast in nonfermentative conditions [16] when artemisinins cause mitochondrial depolarization. Transgenic parasites that no longer rely on mitochondria for asexual stage survival do not show increased resistance to artemisinins, as would be predicted if mitochondria were important targets in vivo [23]. Artemisinins also inhibit the SERCAs of *Toxoplasma* [24, 25] and cancer cell lines in membrane preparations [26] supporting results from PfATP6 [3]. Alternative hypotheses for mechanisms of action of artemisinins continue to be investigated to increase understanding of this important class of drug [8].

In modeling studies, thapsigargin, artemisinins, and thaperoxide [4, 12, 27, 28] may share a common binding site in PfATP6 different from that which binds CPA [29]. Inhibition

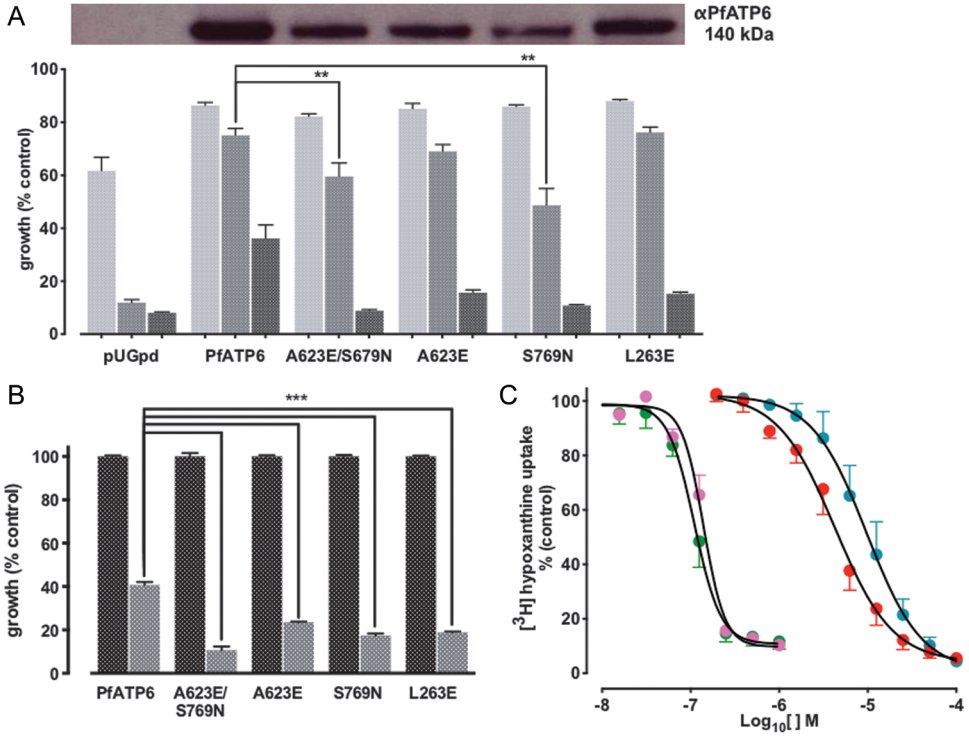

Figure 5. Effect of mutations in K667::*pfatp6* and susceptibility of mutated *P. falciparum* to chloroquine and cyclopiazonic acid. *A*, *Upper panel*: Western blot of total yeast protein. Anti-PfATP6 polyclonal antibody does not detect a band from yeast without PfATP6 (K667::pUGpd), whereas K667::*pfatp6* and mutants express protein. Growth of K667 (assayed as in Figure 3) with vector only or PfATP6 sequences was examined at different concentrations of calcium in yeast peptone dextrose (YPD) and expressed as a percentage of control values after growth without calcium. Data are mean ± SEM values (at least 9 replicates and 3 independent experiments). S769N PfATP6 and A623E/S769N PfATP6 impair growth rescue of yeast in higher Ca^{2+} (≥20 mM) concentrations (PfATP6 vs A623E/S769N $^{**}P = .0079$ PfATP6 vs S769N $^{**}P = .0024$; Student t test). Ten outliers identified by Grubbs test were removed from a total of 504 replicates. *B*, Sensitivity of K667::*pfatp6* wild-type (*dark gray bars*) and *pfatp6* mutants (*light-gray bars*) to CPA (in 20 mM $CaCl_2$). Yeast were assayed and analyzed as in 4a with indicated concentrations of CPA. Inhibition by CPA of PfATP6 wild-type and individual mutants *** $P < .0001$. *C*, IC_{50} values for chloroquine (CQ) and CPA were estimated with [^{3}H]hypoxanthine uptake in growth assays using synchronized ring-stage cultures of *Plasmodium falciparum* (L263 7G8 and L263E 7G8). Growth was measured at 48 h as described by Desjardin et al [50] IC_{50}s ± SEM are the mean of 5 independent experiments (L263 CQ, *green dots*, L263E CQ, *pink dots*, L263 CPA, *blue dots*, and L263E CPA, *red dots*).

by CPA of PfATP6 is demonstrable in 3 systems [3]: after expression in membranes of *Xenopus* oocytes, in yeast assays (this study), and after reconstitution [11]. When CPA inhibition is assayed with the L263E mutation in PfATP6, a compromise in function in yeast increases sensitivity. This is paralleled by increased sensitivity of parasites with the L263E mutation, providing an estimate of the degree of functional compromise (halving of IC_{50} values) caused by such mutations for CPA.

This may explain why transfection of *P. falciparum* with mutated PfATP6, or why field isolates with mutations, yield variable results for susceptibility to artemisinins. Mutations in PfATP6 reduce sensitivity to artemisinins, but because PfATP6 function is compromised, this may not translate to large increases in IC_{50}. A "corrected" IC_{50} value for artemisinins may be obtainable by normalizing results with simultaneous IC_{50} values for CPA.

The L263E mutation may also show less artemisinin resistance in parasites than in oocytes because sequences assayed in oocytes and parasites were not identical and also because of differences between whole cell assays (parasites or yeast) and in *Xenopus* membrane preparations. In parasites, other transport proteins such as PfMDR1 may modulate the artemisinin resistance phenotype [9, 30]. The proposed fitness cost of mutations in PfATP6 may then cause parasites to be outgrown in culture when adapted from patients [31]. Linking mutations in PfATP6 in field isolates to artemisinin resistance could then depend on genetic background, including compensatory mutations in proteins involved in calcium homeostasis. Background is critical to assessing the impact of other resistance genes such as *pfcrt* [32, 33].

The yeast model may therefore allow rapid assessment of functional consequences of mutations in PfATP6, by eliminating variables in parasite assays and overcoming the difficulty of establishing long-term cultures in parasites harboring *pfatp6* field mutations. These mutations may modify the propensity of peroxides to cleave SERCAs through several mechanisms [34]. The increased susceptibility of mutant PfATP6 to CPA (Figure 5),

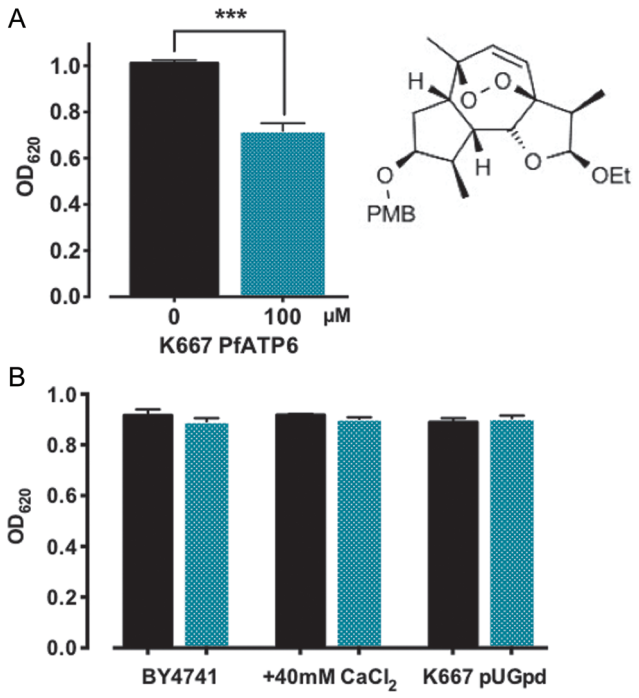

Figure 6. Sensitivity of K667::*pfatp6* to different concentration of thaperoxide 22. *A*, K667::*pfatp6* was diluted to a cell density of 0.1 and inoculated into yeast peptone dextrose (YPD) medium supplemented with 40 mM $CaCl_2$ and thaperoxide 22 as indicated. Yeast cell density was determined after growth at 30°C for 42 h. Data are mean ± SEM of 6 replicates (2 independent experiments). Growth in presence of the drug was compared to controls without drug using Student unpaired *t* test. Inhibition at 100 μM is significant (*** $P < .0001$). The chemical structure of thaperoxide 22 is reported. *B*, BY4741 grown at normal or higher Ca^{2+} concentration and K667 expressing pUGpd are not inhibited by thaperoxide 22 (black bars, no drug; blue bars, 100 μM thaperoxide 22.

in contrast to artemisinins encourages pursuit of CPA derivatives as new leads.

A novel thaperoxide class of antimalarials was developed to test the hypothesis that PfATP6 is targeted by artemisinins. Introduction of a peroxide bridge into the sesquiterpene heart of thapsigargin increases antimalarial potency of thapsigargin by approximately 100-fold. The most potent derivative (thaperoxide 22) also inhibits K667::*pfatp6*, confirming that it remains on target and providing assays for lead optimization (Figure 6). Modeling studies of thaperoxides with PfATP6 and mutants provide testable predictions [35].

Genetic validation of PfATP6 as a drug target should stimulate this further and has been attempted using KO and complementation methods, twice in 2 species of *Plasmodium* (as with PfHT [14]), suggesting that SERCA function is essential for blood stages of infection. Tagging the 3′ end of *pfatp6* and *pbserca* has not been possible despite accessibility of *pfatp6* for single site recombination experiments (Figure 1) [10].

Resistance to artemisinins can be defined as for other classes of antimalarial by a decrease in susceptibility of cultured parasites to one or more derivatives. As resistance increases, it may increase treatment failures of some artemisinin combination therapies. The magnitude of increase in resistance in vitro that is associated with treatment failures in vivo has not been defined [36] although it is noteworthy that some parasites with S769N PfATP6 have IC_{50} values >100 nM for artemether [13, 37]. A requirement for compensatory mutations may have limited the spread of resistance associated with polymorphisms in PfATP6 at present.

An in vivo definition of artesunate resistance that manifests itself in prolonged parasite clearance estimators has been reported with association of this phenotype to parasite's chromosome 13 [38]. This phenotype includes important contributions from host [39] as well as parasite factors in assessment (parasite heritability of approximately 60%), thereby adding to complexities of understanding underlying mechanisms. The contribution of parasite dormancy also needs to be ascertained [40].

Almost 2 decades after P-type cation ATPases were hypothesized to be important drug targets in parasites [41], evidence continues to accumulate in support of SERCAs as targets for artemisinins [8]. Other calcium pumps [5] are now being exploited as targets for new classes of drug such as spiroindolones [42], and the assays presented here may also be useful for their study.

METHODS

Reagents

All reagents were purchased from Sigma Aldrich Chemical Co except for artemisone (made by R. K. H.) and thaperoxide 22 (made by F. S. and M. A.).

Plasmid Construction

An open reading frame of PfATP6 was codon-optimized for expression in *S. cerevisiae* (GeneScript USA Inc). A plasmid containing the wild-type *pfatp6* gene regulated by the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter *TDH3*, was constructed by subcloning the *BamH*I-*Xba*I fragment from commercial vector pcc1 into the expression vector pUGpd (Ura selectable marker) [43]. Vector pUGpd is a yeast centromere plasmid containing a yeast centromere sequence (*CEN*) and autonomously replicating sequence (*ARS*), which confers mitotic and meiotic stability. Mutants of PfATP6 were generated using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene).

Yeast Transformation

The K667 yeast (*Saccharomyces cerevisiae*) strain (*MAT***a**; *pmc1*::*TRP1*; *cnb1*::*LEU2*; *vcx1*Δ; *ura3-1*) [44] was transformed with pUGpd-*pfatp6*, pUGpd-*pfatp6*-L263E, pUGpd-*pfatp6*-

A623E, pUGpd-*pfatp6*-S769N and pUGpd-*pfatp6*-A623E/S769N plasmids, or pUGpd vector only using a Li-acetate method [45]. Transformants were selected on SD medium lacking uracil to yield strains K667::*pfatp6*, K667::*pfatp6*-L263E, K667::*pfatp6*-A623E, K667::*pfatp6*-S769N, K667::*pfatp6*-A623E/S769N, and K667::pUGpd.

Protein Extraction, SDS-PAGE and Western Blotting

SDS gel electrophoresis and Western blots were performed on yeast total cell lysates prepared from equivalent numbers of log phase cells grown in SC-ura medium. Cells were pelleted and lysed by bead beating in SUMEB buffer supplemented with cOmplete ETDA-free Cocktail tablets protease inhibitor (Roche). ER-enriched microsomal membranes were prepared as described elsewhere [46, 47]; 10 μg of protein was used to load gels. Blots were probed with goat anti-PfATP6 antibody, followed by mouse rabbit anti-goat immunoglobulin G (IgG) (H + L)-HRP conjugate secondary (BioRad). Parasite total protein was obtained from saponin freed 3D7 parasites, treated with 0.5% Triton X-100 [48].

Indirect Immunofluorescence of Yeast and Parasites

Polyclonal anti-PfATP6 reagent generated in goat from the peptide CQSSNKKDKSPRGINK (the sequence from Q to K corresponds to the 574–588 region of PfATP6) was used [11]. Immunofluorescence assay (IFA) on K667::*pfatp6* and K667::pUGpd was with anti-PfATP6 antibody as described elsewhere [49]. IFA on *P. falciparum* 3D7 was performed in solution, by fixing infected red cells with 4% paraformaldehyde/0.0075% glutaraldehyde for 30 minutes. Cells were washed in PBS, permeabilized for 10 minutes (0.1% Triton X-100 in phosphate-buffered saline [PBS]) blocked with PBS/3% BSA, and incubated with anti-PfATP6 antibody for 1 hour and then with rabbit anti-goat IgG FITC for 30 minutes. Images were taken using a Nikon TE2000 inverted microscope.

Growth Assays

BY4741 (*MAT***a;** *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) yeast and K667 transformed and untransformed strains were used for growth assays. For spot assays cells were grown in liquid culture overnight at 30°C (in synthetic complete [SC] without uracil and containing 20 g L-1 glucose). After adjusting to OD_{600} = 0.5, 0.1, 0.02, 0.004, aliquots were inoculated onto agar 2% with yeast peptone dextrose (YPD) medium and various additions and incubated at 30°C for 3–5 days. To determine growth rate in liquid $CaCl_2$ solutions, yeast cultures were inoculated in YPD medium and grown at 30°C, shaking for 42 hours in 96-well flat bottomed plates, with absorbance measured at 620 nm. Drug assays were at specified concentrations and growth recorded at plateau phase (18 hours at basal Ca^{2+} concentration and 42 hours at higher Ca^{2+} concentrations). Concentrations between 20 and 40 mM calcium were assayed before performing experiments.

Statistical Analysis

Growth was measured as OD_{620} or as percentage of control (growth in YPD medium only) at different conditions and compared by Student *t*-test (with 2 alpha considered significant at $P < .05$). When groups of 3 drug concentrations were compared with growth without drug (Table 1*A* and Supplementary Table 1*A*), then ANOVA with Dunnett test was applied. Possible outliers were assessed and removed after applying Grubbs test where indicated in results. All analyses were in GraphPad (Prism v6.0a).

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Author contribution. S. K. and H. S. conceived experiments carried out and analyzed by S. P. and K. S., J. K. P. supervised work with yeast, C. D. and J. H. supervised transfection studies with *P. falciparum* and R. T. with *P. berghei*, F. S. and M. A. synthesized thaperoxide, and R. K .H. the artemisinins. All authors contributed to writing the article that was drafted by S. K.

Potential conflicts of interest. S. K. has acted as an advisor to GSK for early drug discovery in the past and S. K. and H. S. are share holders in QuantuMDx. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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