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

Serena Pulcini,1 Henry M. Staines,1 Jon K. Pittman,2 Ksenija Slavic,1 Christian Doerig,3,4 Jean Halbert,4 Rita Tewari,5 Falgun Shah,6 Mitchell A. Avery,6 Richard K. Haynes,7 and Sanjeev Krishna1

1Centre for Infection and Immunity, Division of Clinical Sciences, St. George’s, University of London, United Kingdom; 2Faculty of Life Sciences, University of Manchester, United Kingdom; 3Department of Microbiology, Monash University, Clayton, Australia; 4INSERM-EPFL Joint Laboratory, Global Health Institute, Lausanne, Switzerland; 5Centre for Genetics and Genomics, School of Biology, Queen’s Medical Centre, University of Nottingham, United Kingdom; 6Department of Medicinal Chemistry, University of Mississippi, Oxford; and 7Department of Chemistry, Institute of Molecular Technology for Drug Discovery and Synthesis, Hong Kong University of Science and Technology, Kowloon, (PR China)

Background. The mechanism of action of artesimins 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 artesimins. Mutations in PfATP6 identified in field isolates (such as S769N) and in laboratory clones (such as L263E) decrease susceptibility to artesimins, 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 artesimin resistance may be overcome.

Keywords. Artesimins; 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. Artesimins are key components of most commonly used antimalarial combination therapies. They were hypothesized to act by inhibiting a parasite-encoded P-Type Ca²⁺ 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 artesimins 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 artesimins (reviewed in [6–8]). However, introducing mutations in PfATP6 into cloned parasite lines generates phenotypes that vary in sensitivity to artesimins [9, 10]. Also, highly purified PfATP6 reconstituted in artificial membranes is not inhibited by artesimins and only poorly by thapsigargin, a specific SERCA inhibitor [11] that is predicted in modeling studies to inhibit PfATP6 [12]. To resolve
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 1A), as with the glucose transporter, PfHT [14]. Two attempts were unsuccessful, including complementation with wild-type or double mutated full length pfatp6 (Figure 1C) and tagging the 3′ end of the gene with green fluorescent protein (GFP), myc, or double HA epitopes (Figure 1B). 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 Ca2+ ATPases [15]. This assay has the advantage of assessing drug action in intact cells. The host strain (K667) lacks one of its 2 Ca2+ ATPase genes PMCI, whereas the other gene, PMRI, 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 2A, upper panel) with a similar pattern in parasites (Figure 2A, 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 cmyc 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 Pfhsps86 promoter. Complementation of KO parasites without a tagged pfatp6 construct was not successful, and neither was episomal expression only of PfATP6.
whole cell extracts from yeast by Western analysis (Figure 2B). Endogenous yeast non-SERCA P type Ca\textsuperscript{2+} ATPases (Pmr1p and Pmc1p) in reference strain BY4741 are not detected by anti-PfATP6 anti-peptide polyclonal reagent (Figure 2B, lane a). PfATP6 is detected in membrane extracts from both transgenic yeast and parasites (P. falciparum 3D7; Figure 2B, 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\textsuperscript{2+} ATPases can grow on solid (YPD) medium supplemented with CaCl\textsubscript{2} (0–100 mM, Figure 3A, upper row). As expected, strain K667 cannot grow when stressed with ambient calcium concentrations >25 mM (Figure 3A, 2nd row), and the addition of an empty vector (K667::pUGpd; Figure 3A, 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 3A, last row).

Liquid cultures allow quantitation of growth and show that PfATP6 expressing yeast (OD\textsubscript{620} nm) grow significantly better in media supplemented with ≥50 mM CaCl\textsubscript{2} compared with yeast strains (K667 and K667::pUGpd) without Ca\textsuperscript{2+} ATPases (Figure 3B; P < .001). PfATP6 cannot restore growth in increasing manganese concentrations, confirming that (as with recombinant protein [11]) it acts specifically as a Ca\textsuperscript{2+} ATPase. This manganese sensitivity also confirms that the Ca\textsuperscript{2+}/Mn\textsuperscript{2+} ATPase PMR1 is inactive in K667 (Figure 3C). 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\textsubscript{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\textsuperscript{2+} ATPases [17]. Strain K667 can
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 4A, \(P < .001\)).

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 4A). 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 4B) did not inhibit any strain without PfATP6 (BY4741, K667, K667::pUGpd).

Table 1A 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 1A 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 1A and Supplementary Table 1A). 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.
To confirm that mitochondrial inhibition by artemisinins is not relevant to inhibition of \textit{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 1B) 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 5A, lower panel).

All \textit{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 5A, lower panel). Western analysis confirmed expression of mutant PfATP6 proteins (Figure 5A, 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 1B 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 \textit{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 1B), perhaps reflecting differences in 3 amino acid residues in \textit{PfATP6} sequence expressed in \textit{Xenopus} compared with the wild-type (3D7) \textit{PfATP6} sequence expressed in yeast (sequence differences...
Table 1. Sensitivity of K667 Expressing Wild Type and Mutated PfATP6 to Antimalarials

<table>
<thead>
<tr>
<th>µM</th>
<th>Growth (% control)</th>
<th>PfATP6 wt</th>
<th>A623E/S769N</th>
<th>A623E</th>
<th>S769N</th>
<th>L263E</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µM)</td>
<td>PfATP6</td>
<td>A623E/S769N</td>
<td>A623E</td>
<td>S769N</td>
<td>L263E</td>
<td></td>
</tr>
<tr>
<td>(A) artemisinin</td>
<td>1</td>
<td>100.2 ± 1.1</td>
<td>104.7 ± 1.2</td>
<td>104.3 ± 1.7</td>
<td>105.3 ± 1.7</td>
<td>104.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>87.3 ± 1.2*</td>
<td>104.7 ± 1.7</td>
<td>105.3 ± 1.7</td>
<td>104.4 ± 1.7</td>
<td>104.5 ± 1.9***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>76.4 ± 1.2*</td>
<td>105.3 ± 1.7</td>
<td>104.4 ± 1.7</td>
<td>104.5 ± 1.9***</td>
<td>104.5 ± 1.9***</td>
</tr>
<tr>
<td>artesunate</td>
<td>1</td>
<td>90.1 ± 0.8</td>
<td>97.9 ± 2.0</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>82.8 ± 1.3*</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>72.6 ± 0.8*</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>artemether</td>
<td>1</td>
<td>94.1 ± 2.7</td>
<td>100.1 ± 1.5****</td>
<td>100.0 ± 1.1</td>
<td>100.9 ± 1.5</td>
<td>103.5 ± 1.4****</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>80.7 ± 3.0**</td>
<td>95.7 ± 1.8****</td>
<td>99.7 ± 1.6***</td>
<td>103.0 ± 1.2***</td>
<td>104.2 ± 1.3***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>77.1 ± 2.8**</td>
<td>65.0 ± 4.1</td>
<td>64.6 ± 1.4</td>
<td>74.8 ± 1.6****</td>
<td>69.3 ± 2.4</td>
</tr>
<tr>
<td>artemisone</td>
<td>1</td>
<td>90.0 ± 0.3*</td>
<td>96.6 ± 1.5</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>80.2 ± 0.9*</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>56.3 ± 1.2*</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>DHA</td>
<td>1</td>
<td>80.9 ± 0.9*</td>
<td>96.6 ± 1.5</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>69.1 ± 1.4*</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>65.8 ± 3.0*</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>CPA</td>
<td>1</td>
<td>79.5 ± 6.7**</td>
<td>96.6 ± 1.5</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>31.7 ± 2.4**</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>24.0 ± 1.7**</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>thapsigargin</td>
<td>1</td>
<td>100.5 ± 0.5</td>
<td>96.6 ± 1.5</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>99.5 ± 0.6</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>86.9 ± 0.8*</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>chloroquine</td>
<td>1</td>
<td>101.6 ± 1.4</td>
<td>96.6 ± 1.5</td>
<td>96.6 ± 0.9</td>
<td>91.9 ± 1.1****</td>
<td>98.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>102.3 ± 1.0</td>
<td>91.1 ± 1.1***</td>
<td>86.9 ± 0.6</td>
<td>87.8 ± 1.1</td>
<td>89.0 ± 0.6****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.4 ± 1.2</td>
<td>85.4 ± 0.9***</td>
<td>70.2 ± 2.4</td>
<td>78.5 ± 0.8****</td>
<td>72.2 ± 2.2</td>
</tr>
<tr>
<td>2de-QHS</td>
<td>1</td>
<td>100.9 ± 1.4</td>
<td>100.1 ± 1.5</td>
<td>100.0 ± 1.1</td>
<td>100.9 ± 1.5</td>
<td>103.5 ± 1.4****</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>103.0 ± 1.5</td>
<td>100.1 ± 1.5</td>
<td>100.0 ± 1.1</td>
<td>100.9 ± 1.5</td>
<td>103.5 ± 1.4****</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>98.1 ± 0.9</td>
<td>100.1 ± 1.5</td>
<td>100.0 ± 1.1</td>
<td>100.9 ± 1.5</td>
<td>103.5 ± 1.4****</td>
</tr>
</tbody>
</table>
between PfATP6 in oocytes and the 3D7 sequence can be compared with accession numbers PF3D7_0106300 and the original sequence in [3]). Results (Table 1B) 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 5B) 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 5C 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 6A 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 6B)).

**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
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 IC50 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 artesiminins. Mutations in PfATP6 reduce sensitivity to artesiminins, but because PfATP6 function is compromised, this may not translate to large increases in IC50. A “corrected” IC50 value for artesiminins may be obtainable by normalizing results with simultaneous IC50 values for CPA.

The L263E mutation may also show less artesiminin 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 artesiminin 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 artesiminin 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),
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 IC50 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 artemesunate 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 BamH1-XbaI 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 (MATa; pmc1::TRP1; cmi1::LEU2; vcx1Δ; 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 gel electrophoresis 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 and blocked with PBS/3% BSA, and incubated at 30°C for 3 days. To determine growth rate –0.5, 0.1, 0.02, 0.004, aliquots were inoculated onto agar 2% with 0.5% Triton X-100 [48].

**Indirect Immunofluorescence of Yeast and Parasites**

Polyclonal anti-PfATP6 reagent generated in goat from the peptide CQSSNKDKSPRIGNK (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 (MATa; 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 OD600 = 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 CaCl2 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 Ca2+ concentration and 42 hours at higher Ca2+ concentrations). Concentrations between 20 and 40 mM calcium were assayed before performing experiments.

**Statistical Analysis**

Growth was measured as OD520 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 1A and Supplementary Table 1A), 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**

Acknowledgments. We thank U. Straschil (Imperial College) and D. Guttery (Nottingham University) for essential help with P. berghei infections.

Funding. This work was supported by EU FP7 Marie Curie-funded Initial Training Network InterMal (Grant Agreement No. 215281-2). Work in the SK and CD laboratories was supported in part by the EU-FP7 MALSIG programme and some research leading to these results has received funding from the European Union Seventh Framework Programme ([FP7/2007-2013]) under grant agreement nº 304948 - NANOMAL.

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, and C. D. and J. H. supervised transfection studies with P. berghei. 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.

**References**


