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LETTERS

Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*

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The origin of all mitochondria can be traced to the symbiotic arrangement that resulted in the emergence of eukaryotes in a world that was exclusively populated by prokaryotes[1–3]. This arrangement, however, has been in continuous genetic flux: the varying degrees of gene loss and transfer from the mitochondrial genome in different eukaryotic lineages seem to signify an ongoing 'conflict' between the host and the symbiont. Eukaryotic parasites belonging to the phylum Apicomplexa provide an excellent example to support this view. These organisms contain the smallest mitochondrial genomes known[4,5], with an organization that differs among various genera; one genus, *Cryptosporidium*, seems to have lost the entire mitochondrial genome[6,7]. Here we show that erythrocytic stages of the human malaria parasite *Plasmodium falciparum* seem to maintain an active mitochondrial electron transport chain to serve just one metabolic function: regeneration of ubiquinone required as the electron acceptor for dihydroorotate dehydrogenase, an essential enzyme for pyrimidine biosynthesis. Transgenic *P. falciparum* parasites expressing *Saccharomyces cerevisiae* dihydroorotate dehydrogenase, which does not require ubiquinone as an electron acceptor[8], were completely resistant to inhibitors of mitochondrial electron transport. Maintenance of mitochondrial membrane potential, however, was essential in these parasites, as indicated by their hypersensitivity to proguanil, a drug that collapsed the membrane potential in the presence of electron transport inhibitors. Thus, acquisition of just one enzyme can render mitochondrial electron transport nonessential in erythrocytic stages of *P. falciparum*.

Apicomplexan parasites contain two cytoplasmic organelles endowed with their own genomes—a mitochondrion and a plastid[9,10]. The mitochondrial DNA (mtDNA) encodes just three components of the electron transport chain and scrambled fragments of ribosomal RNA[5,11], thus the mitochondrion requires the import of hundreds of nuclear-encoded proteins not just to serve the physiological functions but also for the upkeep of its separate genetic system. In *P. falciparum*, biochemical data indicate that the mitochondrion is not a source of ATP[12]; furthermore, the genome does not seem to encode critical subunits of the F_oF_1 ATP synthase[13,14]. Yet, the mitochondrial electron transport chain is critical for parasite survival: inhibition of the cytochrome bc_1 complex (Complex III) is the mode of action for a currently used antimalarial drug, atovaquone[15,16]. The parasite encodes at least five mitochondrial dehydrogenases (a rotenone-insensitive NADH dehydrogenase, glycerol 3-phosphate dehydrogenase, dihydroorotate dehydrogenase, succinate dehydrogenase and malate-quinone oxidoreductase); all five enzymes generate reduced coenzyme Q (CoQ), which in turn is re-oxidized by Complex III, feeding the electron transport chain[14] . Dihydroorotate dehydrogenase (DHOD), the fourth enzyme in the pyrimidine biosynthetic pathway, is essential for malaria parasites' survival because they cannot salvage pyrimidines[17]. Although most eukaryotes possess a mitochondrially located, membrane-anchored type 2 DHOD that uses CoQ as the electron acceptor, many bacteria encode a soluble DHOD that is independent of CoQ, using fumarate as the electron acceptor[8] (type 1A, see Fig. 1a). The yeast *S. cerevisiae* seems to have acquired the type 1A DHOD, possibly through a lateral gene transfer from a prokaryote[18]. We cloned the yeast DHOD gene under the control of a constitutive *P. falciparum* promoter in a shuttle vector[19] that permitted generation of stably transfected parasites using the antifolate WR99210 as a selective agent (see Supplementary Fig. 1). To assess the expression of the transgene, it was fused to a gene encoding green fluorescent protein (GFP). As shown in Fig. 1b, RNA encoding DHOD–GFP was detectable by northern blot hybridization and the fluorescent protein could be detected in the cytosol of the transfected parasites, but not in the mitochondria (Fig. 1c). The fusion DHOD–GFP protein was enzymatically active as judged by its ability to rescue a DHOD null *S. cerevisiae* strain, allowing growth under conditions requiring *de novo* pyrimidine biosynthesis (see Supplementary Fig. 2).

We examined the transgenic *P. falciparum* for their susceptibility to atovaquone in a standard 48 h growth inhibition assay measuring ^{3}H-hypoxanthine incorporation. Whereas the parental parasites were fully susceptible to atovaquone with a midpoint inhibition concentration (IC_{50}) of about 1 nM, the transgenic parasites were not inhibited by even the highest concentrations (2,250 nM) of atovaquone (Fig. 2a). Transgenic parasites expressing GFP alone or fused to unrelated genes were equally susceptible to atovaquone as the parental parasites (data not shown), ruling out the possibility that the resistance was due to the expression of GFP or the transfection

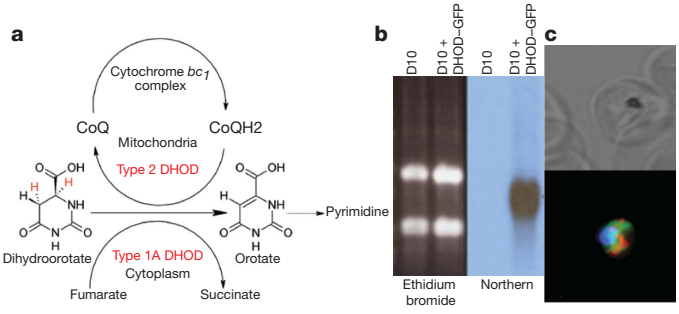

Figure 1 | Transgenic *P. falciparum* expressing *S. cerevisiae* DHOD. **a**, Oxidation of dihydroorotate by Type 1A and Type 2 DHOD is carried out in different cellular locations using different electron acceptors. **b**, The transgene is expressed as a 2.7 kilobase RNA as judged by a northern blot probed with *S. cerevisiae* DHOD DNA. D10 is the parental parasite and D10 + DHOD–GFP is the transgenic parasite line. **c**, The yeast DHOD is localized to the cytoplasm of the transgenic parasite. A phase contrast image of an intraerythrocytic parasite (top), and a confocal image (bottom) of the same parasite showing Mitotracker (red), GFP tag (green) and nuclear (blue) staining.

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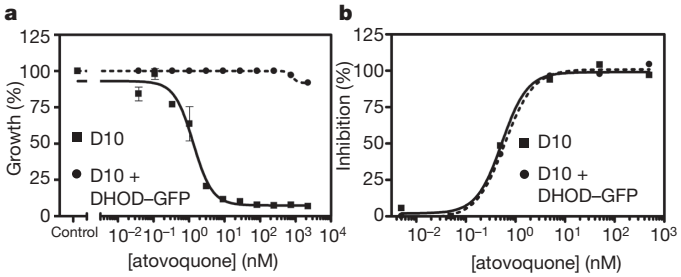

Figure 2 | Transgenic parasites are resistant to atovaquone. **a**, Parasite growth was assessed by ^{3}H-hypoxanthine incorporation when exposed to varying concentrations of drug for 48 h. The parental D10 line was highly susceptible whereas the transgenic D10 + DHOD–GFP was fully resistant to the compound ($\chi^2 = 1.36 \times 10^4$; $P < 0.0001$). Error bars indicate ± s.e.m. ($n = 3$). **b**, The profiles of inhibition by atovaquone of the cytochrome bc_1 complex activity of mitochondria isolated from the parental (D10) and transgenic (D10 + DHOD–GFP) parasites were indistinguishable from each other ($P > 0.999$), showing that the resistance of the transgenic parasites to atovaquone was not due to the resistance of Complex III.

process itself. Because atovaquone resistance in *P. falciparum* can arise quickly both *in vivo* and *in vitro* through point mutations in the mtDNA-encoded cytochrome *b* (ref. 20), we amplified and sequenced this gene from the DHOD-transgenic parasites, but failed to detect any mutations. To examine the possibility that the resistance may be due to mutations elsewhere in the genome, we isolated mitochondria from the parental and transgenic parasites to assess atovaquone inhibition of the Complex III activity and found it to be equally susceptible to atovaquone in both cases (Fig. 2b). We further assessed the susceptibility of the transgenic parasites to other electron transport inhibitors, such as antimycin and myxothiazol, that act at sites different from atovaquone, and found the parasites to be resistant to all these compounds at concentrations > 1,000-fold higher than the IC_{50} for the parental parasites (Table 1). These results suggest that the cytosolic bypass provided by DHOD was sufficient to serve the critical pyrimidine biosynthetic pathway, and that the erythrocytic stages of such parasites became independent of mitochondrial electron transport. At this point, it remains to be tested whether non-erythrocytic life cycle stages of *P. falciparum* could also be rendered independent of mitochondrial electron transport solely through a cytosolic bypass for pyrimidine synthesis. Our results validate the parasite DHOD being investigated as an attractive drug target[21]. On the other hand, the ability of DHOD-transgenic parasites to survive mitochondrial electron transport inhibition suggests that other CoQ-requiring mitochondrial dehydrogenases, such as the single-subunit NADH dehydrogenase and malate-quinone oxidoreductase, are not essential for the growth of erythrocytic stages of *P. falciparum*, and thus not likely to be attractive drug targets.

In the antimalarial drug registered as Malarone, atovaquone is combined with a synergistic partner, proguanil, which we have previously shown to act by lowering the concentration at which atovaquone collapses mitochondrial membrane potential in a rodent malaria model[22]. Proguanil is a prodrug that is converted by a human cytochrome P450 to cycloguanil, which inhibits the parasite dihydrofolate reductase. The synergistic action of proguanil, however, is due to its prodrug form; cycloguanil does not show synergy with atovaquone[22]. We assessed the susceptibility of DHOD-transgenic parasites to proguanil in both the presence and absence of atovaquone. As shown in Fig. 3a, proguanil alone had similar IC_{50} values, ~55 μM, for both the transgenic and parental parasites. Remarkably, when 100 nM atovaquone was included in the medium for the transgenic parasites, the IC_{50} for proguanil was reduced 1,000-fold to 45 nM. Inclusion of 1 μM proguanil decreased the IC_{50} of atovaquone from 1.3 nM to 0.23 nM in the wild-type parasites, but from > 2,250 nM to 0.68 nM in the transgenic parasites (Fig. 3b and Table 1). Similar reductions in IC_{50} values were also seen in the transgenic parasites exposed to 1 μM proguanil and varying concentrations of antimycin and myxothiazol, which by themselves were ineffective against the transgenic parasites (Table 1). These results suggest that proguanil affects a mitochondrial function that becomes essential only when mitochondrial electron transport is inhibited. Maintenance of electropotential across the mitochondrial inner membrane is a critical function of the electron transport chain[23]. When we examined the transgenic parasites treated with atovaquone, mitochondrial membrane potential, as judged by accumulation of a lipophilic cationic compound (Mitotracker) within the mitochondria, was not eliminated, a phenomenon also observed in parental parasites (Fig. 3c). However, the combination of atovaquone and proguanil seemed to be highly effective in collapsing mitochondrial membrane potential. Mitochondrial membrane potential collapse was observable within 15 min of treatment with atovaquone plus proguanil, with 75% of the parasites losing the potential in 2 h of treatment (see Supplementary Fig. 3). Mitotracker staining in these parasites was diffused all throughout the parasite cytoplasm because the intact plasma membrane electropotential[24] drove Mitotracker

Table 1 | Growth inhibition of parental and transgenic *P. falciparum*

Parasite Strain	Proguanil (1 μM)	IC_{50} (nM)			
		Atovaquone	Myxothiazol	Antimycin	Chloroquine
D10	−	1.3	37.2	129.4	87.0
D10	+	0.23	14.9	71.45	62.6
D10 + DHOD–GFP	−	>2,250	>3,300	>33,300	62.1
D10 + DHOD–GFP	+	0.68	21.9	67.44	62.7

The growth of parental (D10) and transgenic (D10 + DHOD–GFP) parasites exposed to various inhibitors was examined by assessing ^{3}H-hypoxanthine incorporation. Midpoint inhibition concentrations (IC_{50}) of the compounds in the absence and presence of 1 μM proguanil were determined.

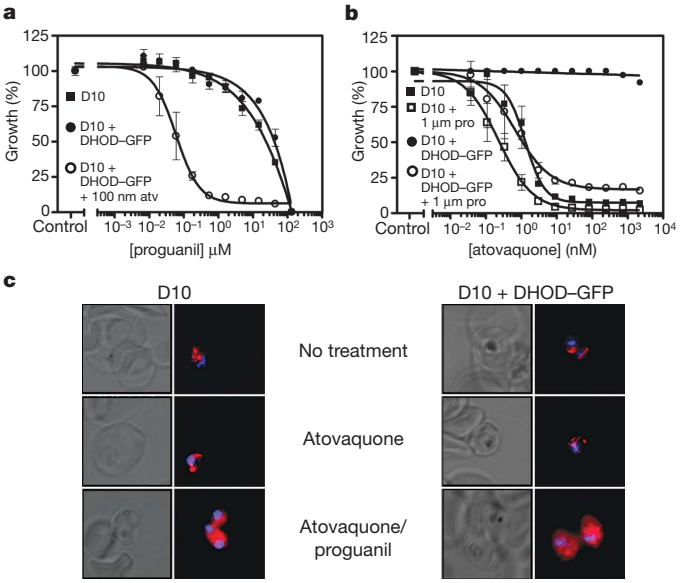

Figure 3 | Extreme hypersensitivity of the transgenic parasites to atovaquone/proguanil combination. **a**, Both the parental and transgenic parasites showed comparable responses to proguanil. Inclusion of 100 nM of atovaquone reduced the proguanil IC_{50} by 1,000-fold in transgenic parasites ($P < 0.0001$). **b**, Inclusion of 1 μM proguanil reduced the IC_{50} of atovaquone by about sixfold in the parental parasites, but by > 3,300-fold in the transgenic parasites ($P < 0.0001$). Error bars indicate ± s.e.m. ($n = 3$). **c**, Mitochondrial membrane potential assessed by the accumulation of Mitotracker is maintained in both the parental and transgenic parasites incubated for 24 h with 100 nM atovaquone. Incubation with atovaquone and proguanil eliminated mitochondrial membrane potential; cytoplasmic accumulation of the probe is driven by the plasma membrane potential of the parasite[23].

accumulation inside the parasite, but the lack of mitochondrial membrane potential precluded its concentration within the mitochondria. These results suggest a proguanil-sensitive pathway for generating mitochondrial membrane potential in malaria parasites that is independent of the electron transport chain.

In many eukaryotes, when electron transport is rendered nonfunctional, mitochondrial membrane potential can be maintained for a period by the reverse action of F_oF_1 ATP synthase using ATP hydrolysis to pump protons[23]. This may be unlikely in *P. falciparum* because mitochondrial ATP synthesis appears to be absent[12], and a complete F-type ATP synthase does not seem to be present[13,14]. Yet, genes encoding parts of the F_1 domain of the enzyme complex, especially the α and β subunits that possess the ATPase activity, are clearly present in *Plasmodium*. Even in *Cryptosporidium*, a genus that has lost mtDNA in its entirety, genes encoding α and β subunits of ATP synthase as well as an ATP/ADP translocator seem to be still maintained. Interestingly, *Cryptosporidium* does not depend on *de novo* pyrimidine biosynthesis, having acquired pyrimidine salvage enzymes through apparent lateral gene transfers[25]. A vestigial mitochondrial structure continues to be maintained in *Cryptosporidium*[26], most probably to serve the generation, assembly and transport of iron–sulphur clusters, an essential pathway that requires electropotential across the inner mitochondrial membrane[27]. We propose that this electropotential is established through a combined action of the matrix-located F_1 sector of ATP synthase and the membrane-located ATP/ADP transporter (see Supplementary Fig. 4 for a model). In our proposal, ATP will be hydrolysed by the ATP synthase and ADP^{3-} generated will be exchanged for ATP^{4-} by the ATP/ADP transporter, resulting in a net negative charge gain for the matrix, thereby establishing the membrane potential, which after all requires the movement of but a few ions across the membrane[23]. A similar means to establish mitochondrial membrane potential has been suggested in *Saccharomyces*[28] and *Trypanosoma*[29] that lack mtDNA. Because the electron transport chain in *P. falciparum* is dominant in establishing the membrane potential, the alternative (proguanil-sensitive) pathway becomes apparent only when electron transport is inhibited. On the other hand, the lack of mtDNA, and thus the electron transport chain, in *Cryptosporidium* makes the alternative pathway for generating membrane potential the only route available. By providing a means to acquire independence from the electron transport chain for membrane potential generation in DHOD-transgenic *P. falciparum*, we may have experimentally achieved a condition that *Cryptosporidium* seems to have achieved through evolution.

The loss of the mitochondrial genome seems to have occurred in many single cell eukaryotes[30]. At the evolutionary scale, Apicomplexan parasites appear to be in the process of minimizing mitochondrial contributions to their physiology. In erythrocytic stages of *P. falciparum*, mitochondrial electron transport can be rendered unnecessary by acquisition of just one metabolic enzyme. It would seem that the only function of mtDNA, which is the provision of a few subunits of the electron transport chain, could be made superfluous by lateral gene transfers that bypass the need for electron transport. The system described here now provides means to investigate what other metabolic adjustments need to be made for the optimal functioning of an organism lacking mitochondrial electron transport.

METHODS

Detailed methods and procedures are provided in Supplementary Information.

Transgene construction. The plasmid pHHMC*/3R0.5 was used as the vector[19]. Genes encoding GFP and *S. cerevisiae* DHOD were PCR amplified, and cloned as a translational fusion into the unique *Bst*B1 and *Xho*I sites of the vector, placing the transcription of the fused gene under the control of a *P. falciparum Hsp86* promoter, yielding the plasmid pHHyDHOD–GFP. The plasmid contains a human dihydrofolate reductase gene as a WR99210-selectable marker.

Parasite culture and transfection. *P. falciparum* clone D10 and its transgenic derivative were cultured in human erythrocytes at 5% hematocrit in RPMI1640 medium containing 0.5% Albumax. Transfection was carried out through electroporation of the purified pHHyDHOD–GFP plasmid into ring stages of *P. falciparum* using a BioRad GenePulser.

Growth inhibition assays. Parasite growth as measured by ^{3}H-hypoxanthine incorporation was assessed by incubation with various doses of inhibitors over a 48 h period.

Parasite mitochondrial isolation and biochemical assay. Mitochondria from parental and transgenic *P. falciparum* trophozoite stages were isolated by a method that employed nitrogen cavitation of parasites, magnetic hemozoin removal and differential centrifugation. See Supplementary Information for details. Cytochrome bc_1 complex activity was assessed by reduction of cytochrome *c* using a synthetic ubiquinol as the electron donor. Inhibition of this activity by atovaquone was assayed at various concentration of the inhibitor.

Microscopic localization and membrane potential assessment. The localization of the DHOD–GFP was carried out by immunofluorescence using an anti-GFP antibody. Membrane potential was assessed by accumulation of MitoTracker (Molecular Probes) as described in Supplementary Information. Confocal microscopic images were obtained with an Olympus system and deconvoluted using SlideBook software.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Supplementary Figures

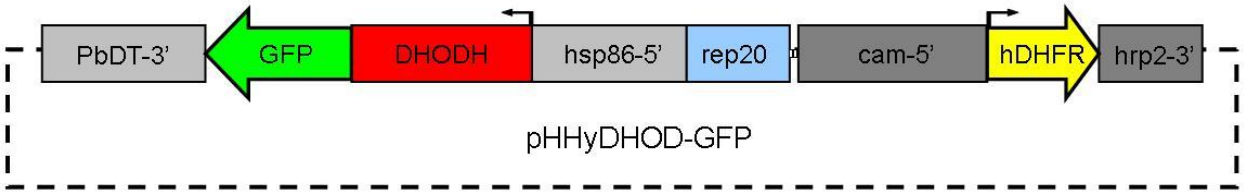

Fig. S1. Configuration of the vector used to introduce *S. cerevisiae* DHODH gene in transgenic *P. falciparum*. The WR99210-selectable hdhfr gene is driven by the constitutive calmodulin promoter, whereas the transgene, fused to GFP, is driven by hsp86 promoter of the parasite. The plasmid also contains a telomere-associated repeat element of *P. falciparum* called rep20, which appears to facilitate segregation of the transgene in replicating parasites[1].

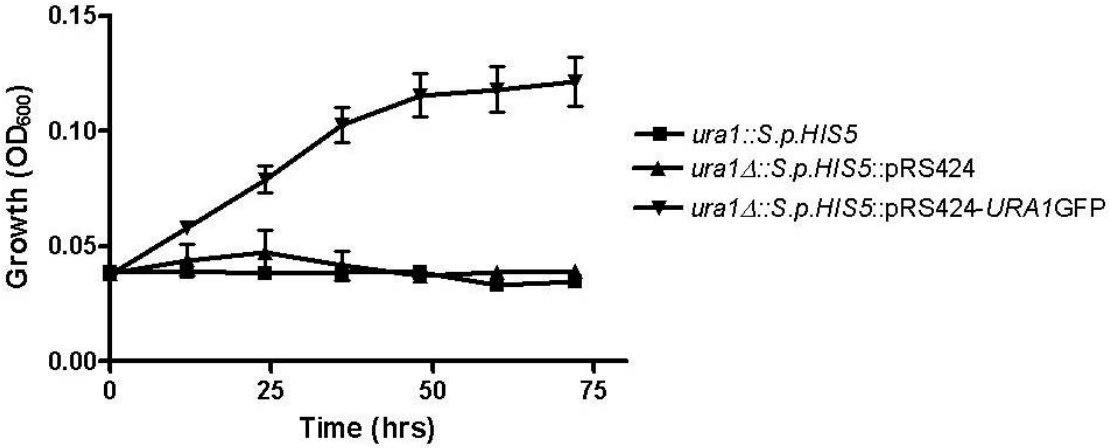

Fig. S2. Genetic evidence for the biochemical function of yDHOD-GFP fusion protein. A *S. cerevisiae* strain (*ura1::S.p.HIS5*) with the deletion of its chromosomal *DHOD* gene (*Ura1*) was derived as described in Supplementary Information. This strain was transformed with either the vector pRS424 (*ura1Δ::S.p.HIS5::*pRS424) or the vector containing the *yDHOD-GFP* fusion gene (*ura1Δ::S.p.HIS5::*pRS424-URA1-GFP). Growth of these three strains in a minimal medium lacking tryptophan and uracil was assessed by measuring optical density at 600 nm. Growth was observed only in the *ura1Δ::S.p.HIS5::*pRS424-URA1-GFP strain.

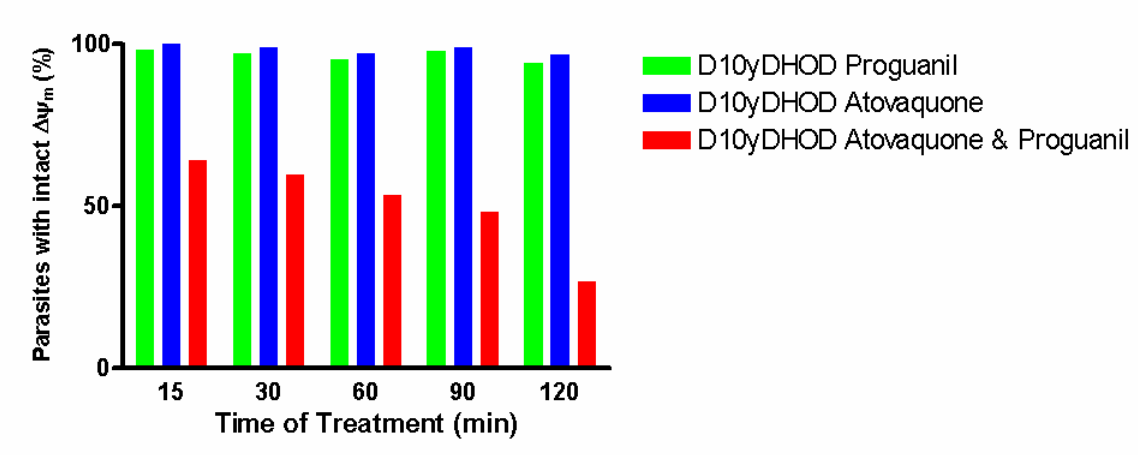

Fig. S3. Mitochondrial membrane potential ($\Delta\psi_m$) in yDHOD-transgenic *P. falciparum*. The transgenic parasites were treated with 1 μM proguanil (green bars). A separate flask of parasites was treated with 100 nM atovaquone for 24 h, divided in two groups, with one getting continued treatment with 100 nM atovaquone and the other treated with 100 nM atovaquone plus 1 μM proguanil. At the indicated time, aliquots were stained with Mitotracker, fixed, and examined by confocal microscopy. Percent of parasites demonstrating $\Delta\psi_m$ were assessed by examining images of at least 100 parasites. Elimination of $\Delta\psi_m$ by atovaquone/proguanil combination was observed in >75% of parasites within 120 min (p=0.0025).

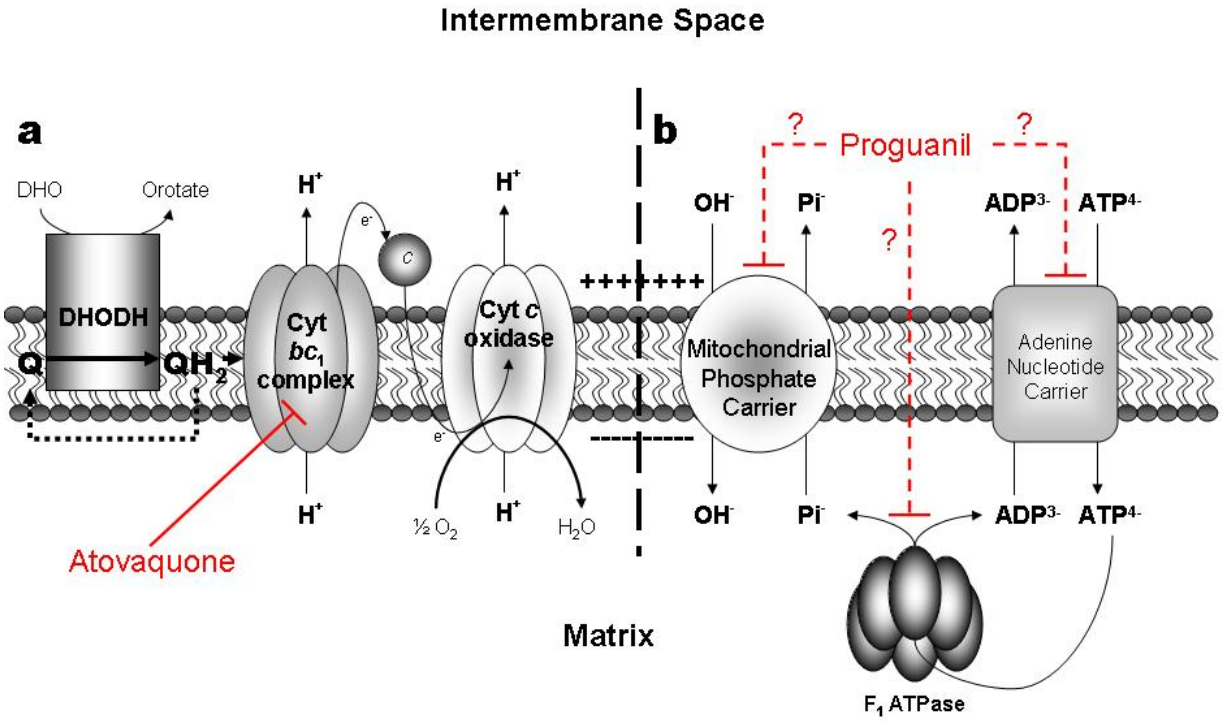

Fig. S4. A model describing the generation of mitochondrial membrane potential in *P. falciparum*. a. The usual mitochondrial electron transport-dependent membrane potential generation involves reduction of CoQ (Q→QH_2) by various dehydrogenases, of which DHOD appears to be the essential enzyme. Re-oxidation of QH_2 by the cytochrome bc_1 complex and subsequent electron transfer to cytochrome *c* and oxygen results in proton translocation and generation of electropotential across the inner membrane with the matrix being negatively charged. Atovaquone, by inhibiting the cytochrome bc_1 complex, will prevent this mode of electropotential generation and will also prevent re-oxidation of QH_2. **b.** Another route for electropotential generation could be through adenine nucleotide carrier (ANC) in conjunction with the F_1 sector of the F-ATPase and the mitochondrial phosphate carrier. Import of ATP^{4-} in exchange for ADP^{3-} by ANC would be electrogenic, producing a net negative charge in the matrix. The imported ATP would be hydrolyzed to ADP and inorganic phosphate (P_i) by the F_1 ATPase, ADP will be exchanged for ATP from the intermembrane space by the ANC, and P_i^- will be exchanged for OH^- by the mitochondrial phosphate carrier (an elecrtoneutral exchange). When membrane potential generation through the electron transport chain is inhibited, this alternate path can provide the necessary membrane potential. Proguanil may interfere with any one of the three components of this alternate system as indicated; in the presence of atovaquone or other electron transport inhibitors, the generation of electropotential would be hypersensitive to proguanil.

Supplementary Methods

Vector Construction. The *Saccharomyces cerevisiae* DHODH gene *ura1* was introduced into the pHHMC*/3R0.5 vector used for *P. falciparum* transfection [1]. This plasmid contains the WR99210-selectable human dihydrofolate reductase (*hdhfr*) gene under the control of *P. falciparum* calmodulin promoter. To assist in determining protein localization, a gene encoding the green fluorescent protein (GFP) was inserted into the XhoI site of pHHMC*/3R0.5. To introduce restriction sites we amplified GFP from pHDGFP[2] using primers SA10GFPXhosen01 (5'- GCT CTC GAG TCT GCA GCA GCA GCA GCA GCA GCA G -3') and SA10GFPSalant02 (5'- GCA GTC GAC TAT TAT AAA TCT TCT TCA GAT ATT AAT TTT TGT TCA GAT CC -3') and ligated the product into the Xho1 site of pHHMC*/3R0.5.GFP was introduced into the pHHMC*3R0.5 expression vector with a mutation at the 3' end of GFP to remove a BstBI restriction site. The resulting plasmid, pHHGFP1.9, was then digested with XhoI and BstBI. *S. cerevisiae URA1* was amplified from strain YKH111 [a kind gift from Dr. K.W. Henry] using primers ScURA1S2BB1F (5'-TAC ACC GCG GTT CGA AAT GAC AGC CAG TTT AAC TAC CAA-3') and ScURA1Xho1R (5'- TAC ACT CGA GAA TGC TGT TCA ACT TCC CAC G-3') and ligated into pHHGFP1.9. This plasmid was transformed into, replicated in, and isolated from DH5α *E. coli* for transfection into *P. falciparum.* The resulting plasmid called pHHyDHOD-GFP is described schematically in Fig. S1.

Parasite culture. P. falciparum clone D10 and its transgenic derivative (D10::yDHOD) were propagated in human erythrocytes by a modification of the method described previously [3]. Cultures were maintained at 5% hematocrit of human erythrocytes in RPMI1640 containing hypoxanthine, NaH_2CO_3, Hepes,glutamine and 5 g/L Albumax.

Transfection of Plasmodium falciparum. Transfection of *P. falciparum* strain D10 was performed as previously described [4]. Briefly, 5-7% ring stage parasite cultures were washed three times with cytomix [5]. The parasitized RBC pellet was resuspended to 50% hematocrit in cytomix. The plasmid isolated using a Qiagen Maxikit (Qiagen, Valencia, CA, USA) and stored in 50 μg aliquots in ethanol was centrifuged down and resuspended in 100 μl Cytomix. The plasmid and 250 μl of the 50% parasitized RBC suspension were combined and transferred to a 0.2 cm cuvette on ice. Electroporations were carried out using a BioRad GenePulser set at 0.31kV, 960uF. The electroporated cells were immediately transferred to a T-25 flask containing 0.2 ml uninfected 50% RBCs and 7 ml medium. To select for parasites containing plasmid, medium containing 5nM WR99210 was added at 48 hrs post transfection. Cultures were maintained under constant 5 nM WR92210 pressure, splitting weekly, until viable parasites were observed.

Inhibitors. The antimalaria compound atovaquone was a gift from Glaxo Wellcome, Research Triangle Park, N.C. Proguanil was kindly provided by the Jacobus Pharmaceutical Company, Princeton, NJ, USA. Mitochondrial respiratory chain inhibitors, antimycin A, myxothiazol, and chloroquine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Growth Inhibition assays. All parasite growth inhibition assays were performed in 96-well plates as described by Desjardin et al. [6]. *P. falciparum*-infected erythrocytes at 1.0% initial parasitemia and 4% hematocrit were exposed to various concentrations of inhibitors in the presence and absence of proguanil for 24 h and then pulsed with 0.5 μCi of ^{3}H-hypoxanthine for 24 h. Incorporation of radioactivity into nucleic acids served as a measure of cell proliferation.

Immunofluorescence imaging. Synchronized ring stage *P. falciparum* D10 or D10::yDHODH infected erythrocytes were incubated for 24 hours with 100 nM atovaquone, 100 nM atovaquone plus 1 μM proquanil, or in the absence of drug. These parasites were then incubated for an additional 30 min in the medium containing 60nM CM-H_2-X-ROS Mitotracker (Invitrogen, Molecular Probes, Carlsbad, CA, USA). The cells were fixed with 4% formaldehyde/0.0075% gluteraldehyde (Polysciences, Inc., Warrington, PA, USA) using the method of Tonkin et al[7]. Fixed cells were permeabilized with 0.05% saponin (Sigma-Aldrich, Inc, St. Louis, MO, USA, cat #S1252), blocked with 10% normal goat serum, 3% BSA, and 0.1% gelatin. To detect yDHOD localization, the suspension was then incubated with a 1:200 dilution of mouse monoclonal anti-GFP antibody (Sigma-Aldrich, Inc., St. Louis, MO, USA) followed by secondary antibody Alexaflour-488 goat anti-mouse (Molecular Probes, Eugene, OR, USA) diluted to 1:250. Cells were further stained with 1.5 μg/ml DAPI (Molecular Probes, Eugene, OR, USA) to stain parasite nuclei, resuspended in Slowfade Antifade reagent (Molecular Probes, Eugene, OR, USA), and mounted on slides using Flouromount-GTM (Southern Biotech, Burmingham, AL, USA). Confocal microscopic images were obtained with an Olympus system and deconvoluted using SlideBook software.

Genetic evidence for the biochemical function of the URA1-GFP fusion protein. In order to test the functional activity of the GFP-tagged *URA1* protein, we carried out complementation of a u*ra1(yDHOD)* deletion strain of *S. cerevisiae* by the URA1-GFP fusion protein. The knockout strain was derived from IPY36 (*MAT*a *his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG pdr5::URA*) provided by Dr. Karl. Henry (Drexel University) and generated by the method described by Longtine *et al*.[8] using a PCR based disruption cassette containing a *Schizosaccharomyces pombe*

HIS5^{+} gene as a selectable marker, resulting in the IPY36*ura1*::*SpHIS5* strain. The fragment containing *URA1-GFP* fusion gene was isolated from the *falciparum* transfection plasmid pHHyDHOD-GFP (Fig. S1) and cloned into the yeast shuttle vector pRS424GPD (containing a *TRP1* selectable marker gene)[9] to generate the plasmid pRS423URA1-GFP. The *ura1* knockout strain IPY36*ura1::SpHIS5* was transformed with either the vector pRS424 or the pRS424URA1-GFP plasmid and selected for growth in a minimal medium lacking tryptophan. Growth of the parental yeast strain IPY36*ura1::SpHIS5* as well as those transformed with either pRS424 or pRS424URA1-GFP was assesses in a minimal medium lacking both tryptophan and uracil.

Preparation of P. falciparum mitochondria. *P. falciparum* cultures were synchronized at least twice by treatment with alanine [10], expanded and harvested at 8-15% parasitemia in the mid to late trophozoite stages. Fractions substantially enriched in mitochondria were prepared using a procedure modified from the method of Takashima *et al.* [11]. Parasitized erythrocytes were harvested by centrifugation, and lysed with 0.05% (w/v) saponin in AIM. (120 mM KCl, 20 mM NaCl, 20 mM glucose; 6 mM HEPES, 6 mM MOPS, 1 mM $MgCl_2$, 0.1 mM EGTA, pH 7.0). After washing 3 times with AIM and once with MSEH (225 mM mannitol, 75 mM sucrose, 4.3 mM $MgCl_2$, 0.25 mM EGTA, 10 mM HEPES [Tris], 5 mM HEPES [KOH]; pH 7.4), the parasites were disrupted by N_2 cavitation (using a 4639 Cell Disruption Bomb, Parr, USA) at 1000 psi for 20 min at 4°C in deaerated MSEH buffer containing 5 mM glucose and mitochondrial substrates (2.5 mM succinate, 5 mM D,L-malate, 2 mM α-glycerophosphate, and 1 mM dihydroorotate) in the presence of 1 mM PMSF and 1 μl per ml fungal protease inhibitor cocktail (Sigma-Aldrich, Inc., St. Louis, MO, USA). After drop-wise release from the N_2 bomb, another aliquot of protease inhibitors was mixed into the disrupted parasite sample. The

unbroken cells and cell debris were removed by centrifugation at 900×*g* for 6 min at 4°C. The low speed supernatant was passed slowly through a MACS CS column prewashed with MSEH in a Vario MACS magnetic separation apparatus (Miltenyi Biotec, Auburn, CA, USA) to remove most of the hemozoin from the preparation. The mitochondria were then recovered as a pellet by centrifugation at 24000×*g* for 20 min at 4°C. The pellet was suspended in a minimal volume of MSEH containing 0.75 mM succinate and used for enzymatic assay or stored at -80°C.

Determination of ubiquinol-cytochrome c oxidoreductase (cytochrome bc_1) activity. Cytochrome *c* reductase activity was assayed by a modification of the method of Trumpower and Edwards [12]. The assay was performed at 35 °C in a stirred cuvette with a final volume of 1 ml containing various amounts of mitochondrial preparation, 100 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone (DBH), 100 μM horse heart cytochrome *c*, 0.1 mg/ml sodium dodecyl maltoside, 35 mM Tricine, 35 mM HEPES, 35mM MOPS (pH 7.4), 10 mM sodium malonate, 1.0 mM EDTA, and 2.0 mM KCN. The reduction of cytochrome *c* was recorded with a modified SLM-AMINCO DW2C dual wavelength spectrophotometer (On-Line Instrument Systems, Inc., Bogart, GA, USA) in dual mode (550 nm – 541 nm). The short chain ubiquinol analog DBH was prepared by reducing 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Sigma-Aldrich Inc., St. Louis, MO, USA) in DMSO with sodium borohydride and acidifying the mixture with concentrated HCl, and stored under argon in aliquots at -80°C.

Supplementary References

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