Supplemental Methods:

Immunofluorescence Imaging. The cells were fixed with 4% formaldehyde/0.0075% glutaraldehyde using the method of Tonkin *et al*. (1) PfDHODH was detected with a 1:300 dilution of mouse anti-PfDHODH antibody (a kind gift from Margeret Phillips, University of Texas Southwestern) followed by secondary antibody Alexaflour-488 goat anti-mouse (Molecular Probes) diluted to 1:250. Cells were further stained with 1.5 μg/ml DAPI (Molecular Probes) to stain parasite nuclei, resuspended in Slowfade Antifade reagent (Molecular Probes), and mounted on slides using Flouromount-GTM (Southern Biotech, Birmingham, AL, USA). Images were obtained using an Olympus BX61 system and SlideBook 6.0 (Intelligent Imaging Innovations).

Comparative Genomic Hybridization: Genomic DNA from *Plasmodium falciparum* cultures D6 and SB1-A6 were collected and purified using the DNeasy kit (Qiagen). A Covaris LE220 was then used to shear gDNA to 1-5kb fragments. Sheared DNA was purified using a Zymo DNA Clean and Concentrator-5 kit and size was verified on a 1% agarose gel. Sheared DNA was primed with a random nonamer at 99°C for 5 minutes and cooled on ice for 5 minutes. Each sample was then mixed with 5ul NEB Buffer 2, 5ul 15x dNTP mix (2:1:1:1, A:T:C:G), 4ul Klenow Fragment (NEB, Cat #M0212S), and the volume was brought up to 50ul with water. Samples were moved to an ozone-free facility and 1.5ul of dUTP-Cy3 or dUTP-Cy5 (Enzo, Cat #ENZ-42501 and #ENZ-42502) was added. A two-hour incubation proceeded in a 37°C heat block, protected from light. Following Klenow treatment, samples were purified using a Zymo DNA Clean and Concentrator-5 kit. Equal nanograms of each sample were hybridized on custom 8x15K microarrays from Agilent Technologies (AMADID 032491, GEO Platform GPL17880) as detailed in Kafsack et al., 2012 (2).

Table S1:

Inhibitor	D6	SB1-A6	D6 C276F	SB1-A6 F276C
6-NH_2Ac (nM)	1.55 ± 0.03	>4,500 (4472 ± 20.67)	46.18 ± 0.29	547.3 ± 0.29
6-NH_2Ac + Proguanil (nM)	0.42 ± 0.28	257.5 ± 0.29	30.16 ± 0.31	170.3 ± 0.18
Atovaquone (nM)	0.10 ± 0.19	>1,500 (9891 ± 0.43)	0.28 ± 0.12	25.91 ± 0.62
Atovaquone + Proguanil (nM)	0.08 ± 0.12	29.82 ± 0.28	0.15 ± 0.06	0.81 ± 0.02
DSM-1 (µM)	0.66 ± 0.06	412.2 ± 0.24	6.76 ± 0.11	10.90 ± 0.12
DSM-1 + Proguanil (µM)	0.71 ± 0.15	191.7 ± 0.13	6.99 ± 0.12	4.84 ± 0.09
ELQ-300 (nM)	20.69 ± 0.15	>4,500 (24492 ± 0.53)	102.7 ± 0.13	749.8 ± 0.13
ELQ-300 + Proguanil (nM)	4.64 ± 0.19	480.8 ± 0.17	51.09 ± 0.16	61.10 ± 0.17
DSM-265 (nM)	9.01 ± 0.04	>4,500 (4477 ± 36)	592.7 ± 0.12	62.17 ± 0.16
Chloroquine (nM)	6.59 ± 0.05	7.39 ± 0.07	7.06 ± 0.12	7.21 ± 0.11

Table S1: Drug susceptibility of various parasite lines: Early ring-stage *P. falciparum* strains D6, SB1-A6, and the CRISPR mutant lines $D6^{C276F}$ and $SB1\text{-}A6^{F276C}$ were evaluated for their susceptibility to the listed mtETC and *Pf*DHODH inhibitors with or without the addition of 1.5 µM proguanil. Chloroquine was included as a positive control for the accurate capture of parasite susceptibility. Parasite survival was determined using a traditional 48 h SYBR-green growth inhibition assay (3) and the IC50 was calculated as an average of three technical replicates (±SD) using GraphPad 8.

Figure S1:

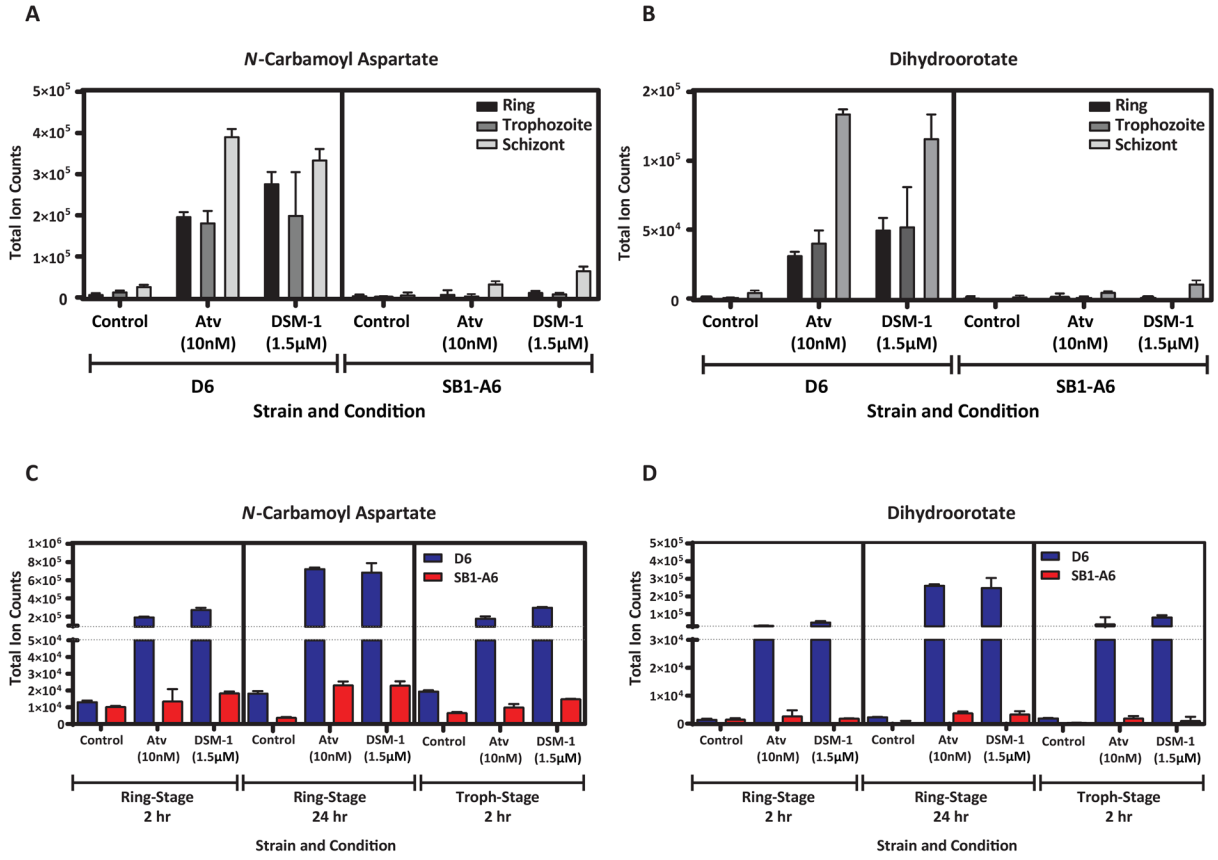

Figure S1: Effects of Inhibitors on pyrimidine synthesis precursors in both sensitive and resistant *P. falciparum*. Flux through *de novo* pyrimidine synthesis was measured in synchronous treated and untreated parasite strains D6 and SB1-A6 by LC-MS. The total ion counts of *N*-carbamoyl aspartate (A) and dihydroorotate (B) were determined at the ring, trophozoite, and schizont stages after two hours of atovaquone (Atv) or DSM-1 exposure at 10x IC_{50}. Similarly, *de novo* pyrimidine synthesis flux was captured after a prolonged 24 h hour exposure to either atovaquone or DSM-1 at 10x $IC_{50,}$ and the total ion counts of *N*-carbamoyl aspartate (C) and dihydroorotate (D) are plotted in comparison to short drug exposures at both ring and trophozoite stages.

Table S2:

Chromosome	Location	GeneID	Description	Mutation
Pf3D7_11_v3	1811743	PF3D7_1145600	conserved Plasmodium protein; unknown function	NON_SYNONYMOUS_CODING (MODERATE|MISSENSE|Gac/Tac|D234Y|629|
Pf3D7_14_v3	1121885	PF3D7_1428500	protein kinase, putative	NON_SYNONYMOUS_CODING (MODERATE|MISSENSE|Gat/Cat|D1003H|2353|
Pf3D7_06_v3	131212	PF3D7_0603300	dihydroorotate dehydrogenase, mitochondrial precursor	NON_SYNONYMOUS_CODING (MODERATE|MISSENSE|tGt/tTt|C276F|569|
Pf3D7_04_v3	676195	PF3D7_0415200	conserved Plasmodium protein, unknown function	STOP_GAINED (HIGH|NONSENSE|Gag/Tag|E982*|2212|
Pf3D7_02_v3	507561	PF3D7_0212400	conserved Plasmodium protein, unknown function	NON_SYNONYMOUS_CODING (MODERATE|MISSENSE|gaG/gaC|E98D|4091|
Pf3D7_14_v3	2588161	PF3D7_1463900	conserved Plasmodium membrane protein, unknown function	NON_SYNONYMOUS_CODING (MODERATE|MISSENSE|aTa/aAa|I510K|1071|

Table S2: SNP analysis of the *P.f.* SB1-A6 genome. The genomes of both the wild-type *P.f.* strain D6 and drug-resistant strain SB1-A6 we examined via Illumina next-generation whole-genome sequencing. SNPs were identified using SNP effector (v 4.3T) (4) and the location of those identified in only *P.f.* SB1-A6 are listed as well as the associated gene and resultant mutation.

Table S3:

Gene ID	Old Gene ID	Probe Name	Description	Log2(D6/SB1-A6)
PF3D7_0602700	PFF0130c	PFF0130C_V7.1_P2/2	conserved Plasmodium protein, unknown function	-0.88
		PFF0130C_V7.1_P1/2	conserved Plasmodium protein, unknown function	-1.19
PF3D7_0602800	PFF0135w	PFF0135W_V7.1_P2/2	JmjC domain containing protein	-0.91
		PFF0135W_V7.1_P1/2	JmjC domain containing protein	-1.18
PF3D7_0602900	PFF0140c	PFF0140C_V7.1_P2/2	conserved Plasmodium protein, unknown function	-0.67
		PFF0140C_V7.1_P1/2	conserved Plasmodium protein, unknown function	-0.96
PF3D7_0603000	PFF0145w	PFF0145W_V7.1_P2/2	SDE2 domain-containing protein, putative	-0.52
		PFF0145W_V7.1_P1/2	SDE2 domain-containing protein, putative	-0.82
PF3D7_0603100	PFF0150c	PFF0150C_V7.1_P2/2	RNA-binding protein, putative	-0.60
		PFF0150C_V7.1_P1/2	RNA-binding protein, putative	-1.13
PF3D7_0603200	PFF0155w	PFF0155W_V7.1_P2/2	mitochondrial chaperone BCS1, putative	-0.84
		PFF0155W_V7.1_P1/2	mitochondrial chaperone BCS1, putative	-1.03
PF3D7_0603300	PFF0160c	PFF0160c_V7.1_P2/2	dihydroorotate dehydrogenase	-0.91
		PFF0160c_V7.1_P1/2	dihydroorotate dehydrogenase	-1.12

Table S3: Copy number analysis of *P.f.* SB1-A6. Genome-wide copy number changes were captured for both *P.f.* strains D6 (Cy3) and SB1-A6 (Cy5) by comparative genomic hybridization using DNA microarray analysis. Fold changes in copy numbers were determined by calculating the fold change (D6/SB1-A6) signal intensity (Log2). Genes are their corresponding DNA microarray probes with an absolute fold change >5 are represented.

Figure S2:

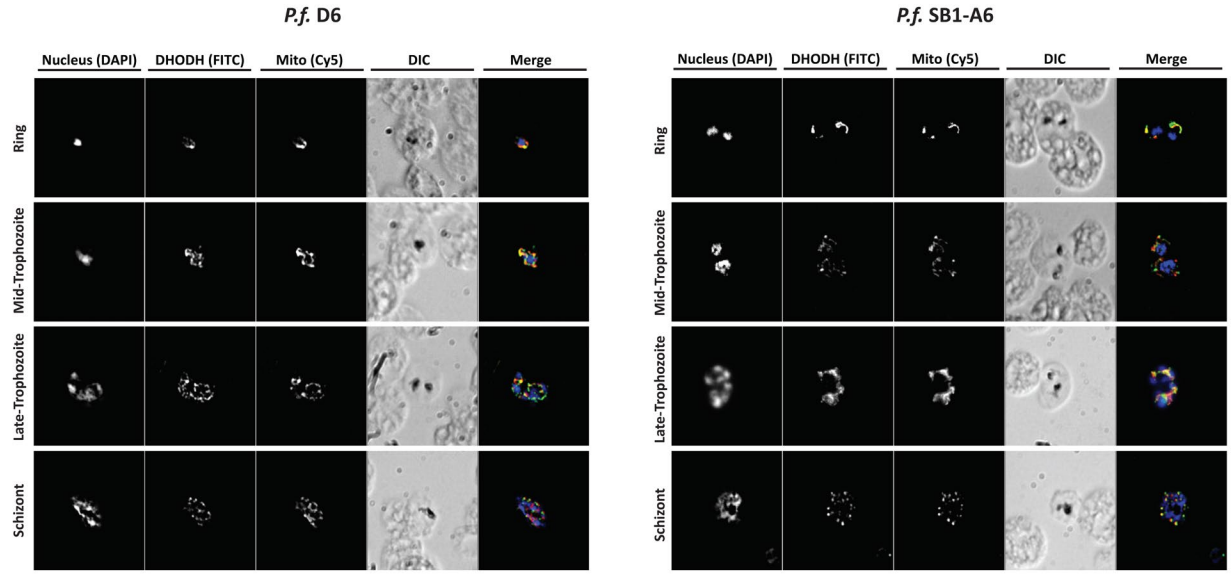

Figure S2: Immunofluorescent Imaging Analysis of *Pf*DHODH localization in both wild-type and drug-resistant parasite strains. Expression and localization of *Pf*DHOD (FITC) to the mitochondrion (Cy5) was visualized by immunofluorescent microscopy; (blue) nuclear DNA stained with DAPI.

Figure S3:

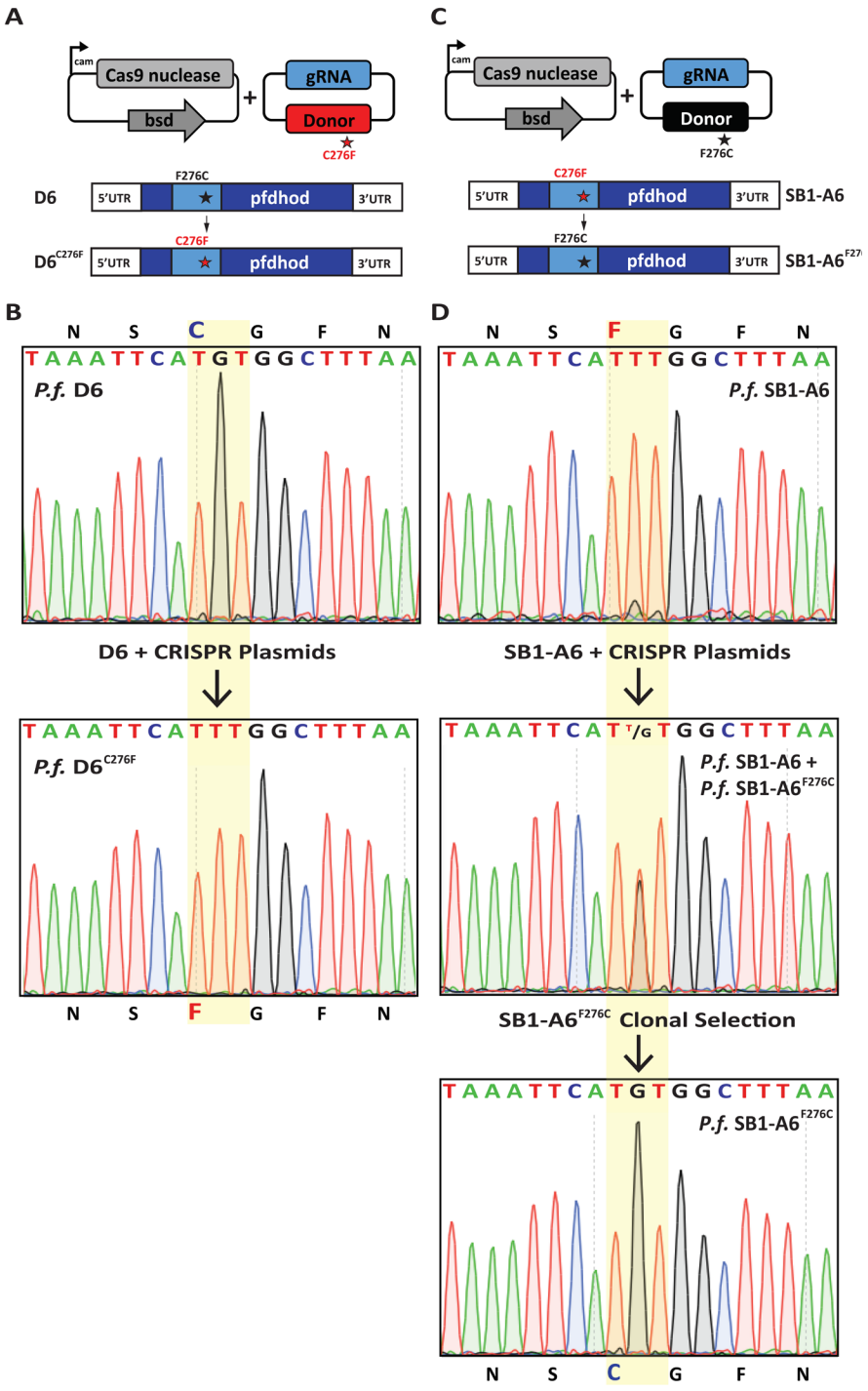

Figure S3: CRISPR-Cas9 editing of *Pf*DHODH. A) Schematic representation of CRISPR-Cas9 mutation strategies for *Pf*DHODH B) DNA sequence chromatogram showing successful mutations of *Pf*DHODH at nucleotide 827 in both C) D6 and D) SB1-A6. The motifs are highlighted in yellow and the PAM is highlighted in green. Mutated nucleotides are underlined.

Supplemental References:

1. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, McFadden GI. 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 137:13-21.
2. Kafsack BF, Painter HJ, Llinas M. 2012. New Agilent platform DNA microarrays for transcriptome analysis of *Plasmodium falciparum* and *Plasmodium berghei* for the malaria research community. Malaria Journal 11:187.
3. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrobial Agents and Chemotherapy 48:1803-6.
4. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 6:80-92.