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Identification and mechanistic understanding of dihydroorotate dehydrogenase point mutations in *Plasmodium falciparum* that confer *in vitro* resistance to the clinical candidate DSM265

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Abstract

Malaria is one of the most challenging human infectious diseases and both prevention and control have been hindered by the development of *Plasmodium falciparum* resistance to existing therapies. Several new compounds with novel mechanisms are in clinical development for the treatment of malaria including DSM265, an inhibitor of *Plasmodium* dihydroorotate dehydrogenase. In order to explore the mechanisms by which resistance might develop to DSM265 in the field, we selected for DSM265-resistant *P. falciparum* parasites *in vitro*. Any of five different amino acid changes led to reduced efficacy on the parasite and to decreased DSM265 binding to *P. falciparum* DHODH. The DSM265-resistant parasites retained full sensitivity to atovaquone. All but one of the observed mutations were in the DSM265 binding site, and the remaining C276F was in the adjacent flavin

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Author Contributions. JW and FEM analyzed resistant *P. falciparum* clones for drug sensitivity and for genetic changes; CLN and JB selected for resistant clones and analyzed clones for drug sensitivity; FEM, AL and GA generated mutant DHODH enzymes and collected enzyme data; XD and DT were responsible for X-ray data collection and structure determination. SKD generated the CRISPR parasite lines and MCSL designed the CRISPR/Cas9 plasmid; MAP supervised enzyme and structural biology studies and analyzed data. DAF and PKR supervised parasite resistance and gene editing studies and analyzed data. MAP wrote the manuscript. All authors reviewed and revised the manuscript.

cofactor site. The C276F mutation was previously identified in a recrudescence parasite during a Phase IIa clinical study. We confirmed that this mutation (and the related C276Y) accounted for the full level of observed DSM265 resistance by re-generating the mutation using CRISPR/Cas9 genome editing. X-ray structure analysis of the C276F mutant enzyme showed that conformational changes of nearby residues were required to accommodate the larger F276 residue, which in turn led to a restriction in the size of the DSM265 binding pocket. These findings underscore the importance of developing DSM265 as part of a combination therapy with other agents for successful use against malaria.

Graphical Abstract

Keywords

Malaria; *Plasmodium*; dihydroorotate dehydrogenase; drug resistance; gene editing

Malaria remains one of the most prevalent global infectious diseases, leading to significant morbidity and mortality.^{1–3} Malaria is endemic in over 90 countries, mostly in the tropics and subtropics, where its transmission by the mosquito host leads to the death of nearly 0.5 million people yearly.⁴ The causative agents of malaria are single-celled protozoan pathogens of the *Plasmodium* genus. *Plasmodium falciparum* is responsible for most malaria deaths while *P. vivax* has the largest global distribution. Sub-Saharan African children under the age of five constitute the population most at risk. There is currently no fully efficacious vaccine and the current bedrocks of malaria prevention and treatment are combination chemotherapies and vector control programs. A number of safe and effective anti-malarial agents have been used clinically since the discovery of quinine over 350 years ago.⁵ However, parasite resistance has compromised the effectiveness of most drugs. The current standard-of-care treatments are artemisinin-based combination therapies (ACTs), which have contributed to the recent decline in malarial deaths over the past decade. However, reduced ring-stage sensitivity to artemisinin derivatives, which manifests as reduced parasite clearance times in patients, combined with emerging resistance to partner drugs, has led to unacceptably high rates of treatment failures in Southeast Asia.^{6, 7}

Drug discovery efforts are underway to identify compounds that can take the place of ACTs should they fail more widely.^{2, 5} All new agents will be introduced as combinations to help avoid resistance. Several entities targeting new mechanisms of action have advanced to Phase II human clinical studies, with the most advanced, artefenomel (OZ439)^{8, 9} and KAF156¹⁰, being clinically evaluated as components of combination therapies. Several

additional compounds have also reached human efficacy assessment, including the dihydroorotate dehydrogenase (DHODH) inhibitor DSM265 (Fig. 1) that was discovered by our group using a target-based approach.^{11, 12} DSM265 showed good safety in a single ascending dose (25–1200 mg) Phase I study in humans, while also demonstrating efficacy in a blood stage human challenge study.¹³ Two human sporozoite challenge studies to evaluate the potential for DSM265 to be used for chemoprevention have also been completed. DSM265 provided full protection from infection if a single dose of 400 mg was given 1 day prior to challenge, while it provided partial protection if dosed 3 or 7 days before challenge.^{14, 15} In a Phase IIa proof of concept study in Peru, DSM265 showed excellent efficacy against *P. falciparum* malaria for patients receiving a single dose of 400 mg (8/9 patients were parasite free as evaluated by the extended 28-day endpoint, with one patient redeveloping parasitemia on day 28).¹⁶ A lower, single dose of 250 mg led to a reduced (60%) cure rate. This study established a single dose of 400 mg of DSM265 as a clinically effective dose when used as a single agent for *P. falciparum*, while efficacy against *P. vivax* was lower. As part of this clinical study recrudescence parasites were sequenced to evaluate the potential for resistance development, and the single *P. falciparum*-infected patient who relapsed on day 28 after receiving the 400 mg dose harbored parasites with a C276Y mutation in the *P. falciparum dhodh* gene. A second patient who received the suboptimal 250 mg dose also relapsed on day 28 and had parasites that contained a mixed population of C276F, G181S and wild-type (WT) *P. falciparum dhodh* with some evidence for *dhodh* gene amplification.¹⁶

As part of our preclinical development program for DSM265, we evaluated the propensity for parasites to become resistant when continually challenged with drug.¹² Parasites selected under drug pressure were also evaluated to determine the mechanism of resistance. We identified both a single point mutation (G181C) and gene amplification of *dhodh* as mechanisms leading to reduced efficacy against *P. falciparum in vitro*. Herein we extend these findings to the identification of additional point mutations, including the selection of a *P. falciparum* Dd2 clone containing the clinically observed C276F mutation, all of which led to significantly reduced *in vitro* efficacy of DSM265. The phenotypes of the C276F and C276Y mutations were validated by introducing these mutations into Dd2 using CRISPR/Cas9 mutagenesis. We generated mutant recombinant enzymes for the amino acid changes identified in the parasite selections, showing that these mutations led to reduced binding to the enzyme. While most of the selected mutations are in the DSM265 binding site, C276 is positioned in the adjacent flavin mononucleotide (FMN) binding pocket, leaving open the question of how mutation of this residue could impact DSM265 binding affinity. X-ray structure analysis of C276F-*PfDHODH* showed that this mutation caused nearby residues to move into the DSM265 binding pocket, providing the structural basis for reduced drug efficacy against this mutant enzyme.

Results

Selection and characterization of DSM265-resistant *P. falciparum* Dd2 cells.

DSM265-resistant Dd2 parasites were previously selected *in vitro* using continuous drug pressure over a range of DSM265 concentrations and variable starting parasite inocula.¹²

Clonal cell lines from R10, R1A and R1B bulk cultures were previously analyzed to determine the shift in DSM265 EC₅₀ (effective concentration leading to 50% reduction in cell proliferation) relative to parental Dd2 parasites, and were also analyzed for sensitivity to atovaquone and artemisinin.¹² To increase representation of possible resistant parasites, we generated clonal lines from two additional DSM265-resistant bulk cultures (R2B and R3B), which had been selected at the same time as the previously described lines. A clonal line from each was isolated and evaluated for drug sensitivities, revealing increases in DSM265 EC₅₀ values of ~15–30-fold (Table 1 and Fig. 2A). Additionally, we recollected drug sensitivity data for our previously reported lines to use as comparators, and observed that the DSM265 EC₅₀ shifts ranged from 20–40-fold (Table 1), similar to our previous report.¹² The observed increases in DSM265 EC₅₀ values were highly significant for all mutant lines (P-values < 0.0001; Table 1). In contrast, the DSM265-resistant parasites retained full sensitivity to atovaquone and artemisinin (Fig. S1 and Table S1).¹² We previously reported that the R10CIB contained a DHODH point mutation (G181C) that correlated with the resistance phenotype. Herein we sequenced the *dhodh* genes from the remaining clones and evaluated *dhodh* gene copy number. Results identified 4 additional DHODH point mutations (C276F, L531F, R265G and E182D), including the clinically identified C276F mutation (Table 1). All clones contained only 1 copy of the *dhodh* gene and we observed no evidence for gene amplification in these DSM265-resistant lines. This is likely explained by the fact that the previously identified gene amplifications (4-fold EC₅₀ shift)¹² were not able to shift the EC₅₀ sufficiently to allow growth under the DSM265 levels (20 – 60 µM; Table 1) used in the selections for the resistant lines described herein.

Evaluation of the effects of mutation on recombinant PfDHODH substrate and inhibitor kinetics.

In order to determine whether the observed point mutations in *PfDHODH* led to a change in binding affinity at the enzyme level, we generated *PfDHODH* recombinant enzymes containing each mutation by site-directed mutagenesis. Additionally, we included C276Y in our analysis, since this was one of the clinically observed mutations.¹⁶ Mutant enzymes were expressed in *Escherichia coli*, purified and analyzed by steady-state kinetic methods (Table 2). Catalytic efficiencies of the mutant enzymes ranged from 0.33 – 0.66 µM⁻¹s⁻¹ with decylubiquinone (coenzyme Q_D; CoQ_D) as the variable substrate, and these values were within 1.5-fold of the wild-type (WT) enzyme (0.44 µM⁻¹s⁻¹). Similar results were previously obtained for a series of alanine mutant *PfDHODH* enzymes (including R265A and L531A) generated in the A77 1726 binding pocket,¹⁷ which overlaps with the DSM265 pocket. However, when analyzing the individual kinetic parameters statistically significant changes were observed in the K_m for CoQ_D for the C276Y and the R265G mutations (4-fold increase). We also observed changes in the K_m for dihydroorotate (DHO) where a 2–3 fold decrease was observed for G181C, L531F and R265G, and a 2-fold increase for the E182D mutation (Table 2). Thus, while the overall catalytic efficiencies for the mutant enzymes were similar to WT, changes in substrate K_m for some of the mutations might impact enzyme activity under conditions where substrate concentrations are at or below K_m.

The effects of the mutations on the IC₅₀ (50% inhibitory concentration) for DSM265 were also evaluated (Table 2 and Fig. 2B). All mutations led to decreased DSM265 potency. For

the G181C, R265G and E182D mutations the IC₅₀ shift was in the 8–33-fold range, values that were within 2-fold of the changes observed for efficacy against the resistant parasite line harboring the same mutation (Tables 1 and 2). The most detrimental mutation at the enzyme level was the R265G mutation (33-fold shift) which also led to a 33-fold reduction in potency on the parasite. Larger differences were observed between the enzyme and parasite data for the L531F mutation, which led to only 2.3-fold increases in enzyme IC₅₀ compared to the 24-fold loss in DSM265 sensitivity versus the R1BC1A parasite cells, and for the C276F mutation where the 37-fold decrease in DSM265 efficacy on the parasite was 5-fold greater than the 8-fold increase in IC₅₀ observed at the enzyme level.

Location of observed mutants within the PfDHODH ligand binding sites.

The observed point mutations were mapped onto our previous X-ray structure of *PfDHODH* bound to DSM265 (Fig. 3). This analysis showed that all but the C276Y/F mutations map to the DSM265 binding site, and these residues are in direct contact with DSM265 within the 4 Å shell. Three of the mutated residues in our resistance selections (E182, R265 and L531) are conserved in DHODH sequences in mammalian and other *Plasmodium* species (Fig. 4; a full sequence alignment is published in¹²), whereas C276 and G181 are variable. Interestingly, C276 is a tyrosine in the mammalian enzymes, similar to that found in one of our resistant clones. Of particular note is the observed mutation of R265. This residue forms a key H-bond interaction with the pyridine nitrogen of DSM265 and its mutation would be predicted to have a significant impact on binding. In contrast, C276 is positioned 6.9 Å away from DSM265 and does not form a direct interaction with the inhibitor. Instead C276 forms part of the FMN and orotate binding sites, which are adjacent to but distinct from the DSM265 binding site.

X-ray structure determination of C276F PfDHODH bound to DSM1.

In order to understand the structural basis for how mutation of C276 could impact DSM265 binding we solved the X-ray structure of C276F *PfDHODH*. To identify an inhibitor for co-crystallization with the mutant enzyme we sought to identify a compound that was not impacted by the C276F mutation. We tested the DSM265-related triazolopyrimidine DSM1¹⁸ because it lacks a substituent in the C2 position (Fig. 1). DSM1 showed a similar IC₅₀ on both WT and C276F *PfDHODH* (IC₅₀ 0.037 ± 0.005 μM for WT versus 0.067 ± 0.02 μM for C276F using the direct assay) and was thereby used in co-crystallizations with C276F *PfDHODH*. The identified crystals displayed symmetry consistent with the P64 space group and diffracted to 2.1 Å. The structure was solved by molecular replacement to an R_{work}/R_{free} = 0.178/0.205 (Table 3), with good electron density for both the DSM1 inhibitor and for C276F (Fig. S2). The C276F-DSM1 structure aligned closely with our prior DSM265 structure (RMSD = 0.28 over 2276 atoms) (Fig. 3). The active site residues that were not adjacent to F276 were also closely aligned, with the exception of M536 that showed movement to accommodate the differences in size between the DSM1 naphthyl and the DSM265 SF₅-aniline. However, we observed significant repositioning of two residues near F276: I263 rotated away from F276 to accommodate the larger residue in the pocket, and E182 also moved to enlarge the space around F276 relative to what was observed in the WT C276 structure. The consequence of the I263 rotation was to move this residue into the CF₂CH₃ binding pocket of DSM265 (substituent on the C2 portion of the ring). Thus, the

structural data show that the C276F mutation led to a rotation of I263 towards the DSM265 pocket, creating a smaller pocket that restricts binding of compounds that have a C2 substituent. Binding of DSM1 that has a hydrogen in the C2 position in contrast is not impacted by these structural changes.

CRISPR/Cas9 edited Dd2 parasites confer resistance to DSM265.

To genetically test the hypothesis that the observed point mutations in *PfDHODH* drive *P. falciparum* resistance to DSM265, we developed a CRISPR/Cas9 editing system to introduce the C276F and C276Y mutations into this gene (Fig. 5; see Methods). These mutations were chosen because in addition to the observation that C276F arose from DSM265 selections of Dd2 parasites, both mutations were observed in clinical isolates.¹⁶ We selected two clones for each variant for subsequent *in vitro* drug susceptibility profiling against DSM265. These clones were denoted as Dd2^{Dhodh} C276F CL1, Dd2^{Dhodh} C276F CL2, Dd2^{Dhodh} C276Y CL1, and Dd2^{Dhodh} C276Y CL2. These edited clones also harbored silent binding-site (BS) mutations to ensure that the edited parasites could not be further cleaved. These BS mutations also provide definitive evidence that these parasites resulted from gene editing events and were not spontaneous mutants. In these assays, we also included the unmodified Dd2 parental line and a CRISPR/Cas9-edited isogenic “BSmut” control line (denoted as the Dd2 BSmut control) that encoded the silent BS mutations and the wild-type amino acid sequence (C276).

The *in vitro* 72 h drug susceptibility assays on Dd2 parasites expressing either the *PfDHODH* C276F or the C276Y allele exhibited high level resistance to DSM265 (Fig. 5B,C). Results from 5–12 independent assays revealed a ~74-fold and a ~24-fold shift in the EC₅₀ values for the C276F and the C276Y variants, respectively, compared with the Dd2 BSmut control ($P < 0.0001$, two-tailed Mann Whitney *U* test; Fig. 5; Table S3). The dose response titrations for the C276F mutant are likely limited by DSM265 solubility at the high concentrations (DSM265 solubility limit at neutral pH ~ 30 μM ¹¹) explaining the shape of the curve and the plateau before 100% inhibition was reached (Fig. 5B). The fold increase for C276F is on par with the 37-fold EC₅₀ increase observed with the DSM265-selected Dd2 R1ACIB C276F mutant parasite (Table 1), confirming that this mutation in *Pfhdh* was the primary driver of DSM265 resistance. High-level resistance imparted by these mutations is consistent with the earlier observation of these mutations in two *P. falciparum*-infected patients who received a single dose of DSM265.¹⁶

Discussion

The development of drug resistance in the field is one of the greatest challenges to maintaining effective anti-malarial drugs.^{3, 6} An important consideration for all new therapies is to evaluate the propensity and the mechanism by which *P. falciparum* acquires resistance, so as to gain insights into the best strategies to protect a new agent as the program moves forward for clinical development. These basic studies also provide markers to monitor the possible emergence of resistance in the field. We undertook *in vitro* resistance selections at subtherapeutic levels using the clinical candidate DSM265 to assess the mechanisms by which resistance may develop. We identified five different amino acid

changes in the active site of the DSM265 target enzyme DHODH, any of which led to decreased efficacy of DSM265 against the parasite. These mutations were also shown to decrease the potency of DSM265 towards recombinant *Pf*DHODH, providing a direct link between the enzyme target and the observed resistance in parasites. The clinically identified mutations C276F and C276Y were validated by introducing them via gene editing into a wild-type *P. falciparum* strain and documenting a gain of resistance, thus conclusively showing that these mutations were the direct cause of reduced efficacy in the parasite. DSM265 and other triazolopyrimidine DHODH inhibitors have previously been shown to have an on-target parasite killing mechanism through the use of a genetically modified *P. falciparum* cell line that expresses yeast DHODH, which is resistant to inhibition by these compounds.^{11, 19, 20} The resistance studies described herein provide additional validation that the mechanism of DSM265 cell killing is through on-target DHODH inhibition. From the clinical perspective, these studies provide a diagnostic marker to monitor for clinical resistance by demonstrating that changes to DHODH will be the primary route to resistance. Clonal parasite lines were also generated that allowed us to characterize the consequence of the clinically observed mutations (C276Y and C276F)¹⁶ on DSM265 efficacy since we were earlier unable to adapt the clinical isolates to *in vitro* culture.

The observation that resistance is selected for by mutations in the DSM265 binding site provides a direct structural basis for reduced efficacy of DSM265 on these parasites. With the exception of C276 (noted above), all of the other mutating residues associated with resistance are within 4 Å of DSM265 in the binding site (Fig. 3). In contrast, C276 is in the adjacent FMN binding site where it is not in direct contact with DSM265. Nonetheless, by determination of the X-ray structure of C276F-*Pf*DHODH we were able to show that this mutation causes conformational changes in adjacent amino acids that impact the binding pocket for C2 substituents on the triazolopyrimidine ring, providing a direct structural explanation for the reduced binding affinity of DSM265 to C276F-DHODH.

The DSM265 binding site is thought to overlap the binding pocket of CoQ based on prior observations that 1) DSM1 is a competitive inhibitor of CoQ and 2) that it inhibits the CoQ-dependent oxidative half-reaction, but not the DHO-dependent reductive half-reaction.^{17, 18} In most enzymes, the active site is highly conserved across species, but in DHODH the inhibitor binding site/presumed CoQ site is highly variable between species.²¹ This variability allows for the identification of highly selective inhibitors, thus avoiding mammalian cell toxicity.^{12, 22} Plasticity in the residues that are compatible with activity allows mutant enzymes to retain enzyme activity.¹⁷ Two of the five identified mutations were in non-conserved residues (G181, C276), though the C276Y change converts the *Pf*DHODH sequence to a residue that is observed in other species including humans. The other three observed mutations were in conserved residues (E182, L531 and R265). Despite the conserved nature of these residues, their mutation had modest impact on the steady-state kinetics of the reaction, as previously observed for characterization of the effects of site-directed alanine mutants in *Pf*DHODH within the inhibitor binding site.¹⁷ While the effects of these mutations on catalytic efficiency are relatively modest, the increased K_m for CoQ for R265G and C276Y shows that these mutant enzymes may be less efficient at substrate concentrations at or below K_m .

The associations between enzyme and parasite efficacy relative to the resistant lines were further confirmed by kinetic analysis of recombinant enzymes containing the observed mutations. The identified mutations led to similar changes on both the enzyme and parasite relative to the wild-type counter parts for most mutations (within 2–3-fold for G181C, E182D, R265G and the C276Y CRISPR-generated line). Larger differences were observed for C276F (5–10-fold if considering both selected and CRISPR-generated lines) and most strikingly for L531F where only a 2.3-fold increase in IC_{50} was observed on the enzyme relative to a 24-fold effect on the parasite. It is important to note that a number of factors can contribute to differential effects of these mutations on the parasite and enzyme. The recombinant enzyme used for *in vitro* enzyme analysis is truncated to remove the N-terminal mitochondrial membrane span; thus the effects of any given mutation might differ within the context of the full-length membrane bound enzyme *in situ*. The relative IC_{50} will also depend on the ratios of substrate concentration to K_m , and whereas the CoQ concentration in the enzyme assay is controlled the intracellular concentration is unknown. Differences in enzyme turnover rates or expression levels for mutant enzymes vs the WT in the parasite might also lead to more pronounced effects in the parasite than on the enzyme. For the C276F, the genetic recapitulation provides conclusive evidence that this DHODH mutation is responsible for DSM265 resistance. Given the small effect of the L531F mutation on the enzyme it remains formally possible that the L531F line may harbor additional changes that further impact DSM265 efficacy in the parasites beyond this mutation. However, the E182D and L531F mutations were also previously identified in selections using different DHODH inhibitors with overlapping binding modes to DSM265^{23, 24}, including a related triazolopyrimidine DSM74²⁵, and an unrelated chemical scaffold Genz669178²⁶ (thiophene-2-carboxamide), providing further validation of the direct link between these mutations and resistance.

The C276 mutations are of particular interest given their selection during the Phase IIa clinical study in Peru.¹⁶ Using three independent methods including parasites selected for resistance, CRISPR/Cas9 generated parasites, and evaluation of these mutations with recombinant enzymes, we confirmed herein that mutation of C276 leads directly to DSM265 resistance in the parasite. The C276Y mutation is associated with a 24-fold higher EC_{50} against the parasite. This change is more than sufficient to explain the lack of parasitological cure within the established safe dosing margins established in humans.^{13, 16} Interestingly, selection of mutations at this residue only occurred at the highest parasite selection numbers (2×10^9) combined with the highest selection concentration (60 nM). This suggests that clinical resistance was difficult to achieve at the clinically effective dose for treatment of 400 mg since the other mutations that were selected *in vitro* under less stringent conditions did not arise. It will be of interest to see if this finding would also be observed in studies with larger cohort sizes. Consistent with the idea that the other mutations occur only at sub-efficacious dosing regimens, a mixed infection (wild-type, C276F, G181S and evidence for gene amplification) was isolated from one recrudescence parasite in the 250 mg cohort.

Importantly, none of the identified mutations led to loss of sensitivity to atovaquone or artemisinin, and all selected parasite lines remained fully sensitive to both. Previously reported selections for resistant parasites with DSM1 led to *dhodh* gene amplification.²⁷ Subsequently a second round of DSM1 selections was then performed on the initial resistant

line resulting in a further increase in gene copy number.²⁸ The resultant cell line exhibited a partial tolerance for atovaquone though these parasites retained full sensitivity to atovaquone/proguanil. In contrast, in our current study we observed no evidence for cross-resistance with atovaquone in our DSM265-resistant mutants, consistent with a distinct mode of action.

Conclusion.

The studies described herein show that resistance to DSM265 can be generated by more than one type of mutation in the binding pocket through selections of parasites under continual DSM265 pressure. These results highlight the importance of developing DSM265 as part of a combination therapy with another anti-malarial agent to protect the target from development of resistant mutants in the field. These studies provide further support for the concept that if DSM265 were developed for treatment (as opposed to chemoprevention), then it would best be paired with a fast-acting compound with gametocytocidal activity that would rapidly reduce the parasite burden and prevent formation of sexual stage parasites required for transmission, thus reducing the potential for resistance to develop and spread.²

Methods

Compound supplies.

DSM265 (2-(1,1-difluoroethyl)-5-methyl-[*N*-[4-(pentafluorosulfanyl)phenyl]1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine)¹² and DSM1 (5-methyl-*N*-(naphthalen-2-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine)¹⁸ were synthesized as previously described. Artemisinin, atovaquone and DHODH substrates decylubiquinone (CoQ_D) (2,3-Dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) and DHO were purchased from Sigma Aldrich (St. Louis, MO.).

Gene sequences.

The P. falciparum dhodh sequence was obtained from PlasmoDB (PF3D7_0603300).

Isolation of DSM265-resistant *P. falciparum* Dd2 clones, gene copy number determination, and sequencing.

Asynchronous cultures of the cloned *P. falciparum* Dd2 parent strain were continuously challenged with DSM265 to obtain drug-resistant parasite lines as previously described.¹² Drug sensitivities of three mutant lines (R10CIB, R1BCIA and R1ACIB) tested with DSM265, atovaquone and artemisinin were previously reported.¹² Two additional lines (R2B and R3B) were cloned from bulk cultures arising from these same selections for evaluation in the present study. All strains were propagated in RPMI-1640 containing 0.5% Albumax II and 2% hematocrit (Type A+ RBCs).^{29–31} For targeted *dhodh* sequencing we employed two methods. In the Rathod lab, DNA was extracted from saponin-lysed parasites using the DNeasy kit (Qiagen) and the *P. falciparum dhodh* gene was PCR amplified using BIO-X-ACT (Bioline) with forward primer (CATTTAAGCCCCAAAACATTTTAC) and reverse primer (GTGATAGATAGCTCCAGTCGATTTC). In the Phillips lab, DNA was extracted by parasite lysis with 0.05% saponin using DNAzol (MRC Inc.), and the *dhodh*

gene was PCR amplified using Phusion (Invitrogen) with forward primer (CATTTAAGCCCCAAAACATTTTAC) and reverse primer (GTGATAGATAGCTCCAGTCGATTTC). All PCR products were either sequenced directly or gel purified, cloned into the TOPO TA vector and then sequenced. TOPO-TA-specific M13 forward and reverse primers were used for sequencing.²⁷ Gene copy number was determined in resistant parasites as previously described.²⁷ In brief, 1 ng/μL of DNA was used per qPCR reaction. *dhodh* forward (TCCATTTCGGTTGTTGCTGCAGGATTTGAT) and reverse primers (TCTGTAACTTTGTCACAACCCATATTA) were used to generate a 206 bp amplicon. Single copy gene control amplicons included seryl tRNA synthetase (PF07_0073) and 18S ribosomal RNA (MAL13P1.435). A melting curve for each primer pair ranging from 55°C to 85°C (0.5°C steps, 1 sec each step) was done at the end of each qPCR run to ensure single amplicon production.

P. falciparum growth EC₅₀ Determination.

Asynchronous cultures were propagated in RPMI media supplemented with human red blood cells to 0.5% hematocrit and 0.5% parasitemia. Drugs were prepared in DMSO as a 500x dilution series (typically a 2 or 3 -fold dilution series was used, yielding a final concentration in media ranging from 0.001 – 30 μM depending on the cell line) and were then diluted 1:50 into media to yield a final DMSO concentration of 0.2%. Parasites were grown at 37°C for 72 h and growth was assessed using the SYBR Green method³² with minor modifications.³³ SYBR green fluorescence was measured (ex./em. 485/535nm) and data analysis was performed using GraphPad Prism 7. Prior to determining EC₅₀, parasites were propagated for three intraerythrocytic cycles in media lacking drug before plating. All data were collected in triplicate.

P. falciparum limited dilution cloning.

Parasite cloning was performed as previously described³⁴ to obtain genetically pure DSM265-resistant lines. Ten or fewer parasitized RBCs per mutant were plated across 96 wells of a microtiter plate. Media and fresh compound were supplied three times per week. Wells positive for parasites were identified by sampling a small volume of red blood cells (RBCs) from each well every media change, staining for parasites with SYBR Green I and processing through a BD Biosciences Accuri C6 flow cytometer. Selection pressure was maintained throughout the cloning process and subsequent expansion into T25 flasks.

PfDHODH constructs for kinetic analysis.

For kinetic analysis, recombinant His₆-PfDHODH (amino acid residues 158–569, where the N-terminal PfDHODH residues start at¹⁵⁸FESYNP) wild-type and mutant enzymes were expressed as an N-terminal truncation, which removes the mitochondrial membrane spanning domain leading to generation of a soluble enzyme as described.^{17, 35} PfDHODH G181C, L531F, R265G, E182D, C276F and C276Y mutants were constructed by PCR mutagenesis of the *pfDHODH*-pRSETb wild-type expression plasmid using the Quick-Change site-directed mutagenesis kit (Stratagene) with the following primers (altered base in bold) and an annealing temperature of 77°C. For simplicity only the forward primers are shown. The resultant mutant *dhodh* genes were sequenced in their entirety.

G181C: 5'-GTT TGA AGT ACA TCG ATT GTG AAA TTT GCC ATG ACC TG-3'

L531F: 5'-GTC AGC TCT ATT CGT GCT TTG TTT TCA ACG GTA TG-3'

R265G: 5'-GAA ACC GCG GAT TTT TGG TGA CGT CGA ATC TCG-3'

E182D: 5'-GTT TGA AGT ACA TCG ATG GTG ACA TTT GCC ATG ACC TG-3'

C276F: 5'-GC TCA ATT ATC AAC TCA TTT GGC TTT AAT AAT ATG-3'

C276Y: 5'-GCT CAA TTA TCA ACT CAT ATG GCT TTA ATA ATA TGG G-3'

***Pf*DHODH constructs for crystallography.**

For crystallography, the pET28b-*p*DHODH 384–413 C276F mutant was generated by site-directed mutagenesis as above using pET28b-*p*DHODH 384–413 wild-type construct^{22, 26} as the template. The 384–413 truncation was previously shown to facilitate crystallization by elimination of a protease sensitive loop.^{22, 26} These constructs also contain the N-terminal truncation of the mitochondrial membrane spanning domain as described above.

Mutagenesis primers were as follows:

GAATCTCGCTCAATTATCAACTCATTTGGCTTTAATAATATGGGTTGCGA, and
TCGCAACCCATATTATTAAAGCCAAATGAGTTGATAATTGAGCGAGATTC.

Protein Expression and Purification.

BL21-DE3 *E. coli* phage - resistant cells containing WT or mutant *Pf*DHODH-pRSETb constructs were grown in Terrific Broth with 10% glycerol and 100 µg/ml ampicillin overnight at 37°C as previously described.¹¹ Cultures (12 L) were inoculated with an overnight culture into the Terrific Broth with 10% glycerol and 100 µg/ml ampicillin and grown at 37°C to an OD₆₀₀ of 0.6–0.8, supplemented with 0.1 mM FMN, then induced with 0.2 mM IPTG at 16°C overnight. Cells were pelleted by centrifugation at 4000 rpm at 4°C and the pellet was resuspended in lysis buffer A (50 mM Tris, pH 8.5, 5 mM 2-mercaptoethanol, 2% Triton X-100, 0.5 mM FMN, 10 % glycerol, 20 mM imidazole pH 8.0, with Protease Inhibitor Cocktail (Sigma P8849) at a 100x dilution. Cells were lysed by three passes through an Emulsi-Flex-C5 high pressure homogenizer. The lysate was then clarified by centrifugation at 20,000 rpm at 4°C, and the clarified supernatant loaded onto a 5 ml HisTrap HP column, pre-equilibrated with HisTrap buffer (20 mM Tris pH 8.5, 300 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.0, 0.05% Triton X-100). Protein was eluted from the column using a linear gradient from 20 to 400 mM imidazole. Fractions containing *Pf*DHODH WT or mutant enzymes were concentrated and purified by gel filtration chromatography on a HiLoad 16/600 Superdex 200 column equilibrated with gel filtration buffer (100 mM HEPES, pH 8.0, 300 mM NaCl, 0.05% Triton X-100 reduced, 1 mM DTT). *Pf*DHODH purity was >95% as determined by SDS-PAGE analysis. DHODH concentration was determined based on the absorbance of FMN at 454 nm ($\epsilon_{445} = 12.5 \text{ mM}^{-1} \text{ cm}^{-1}$).¹⁷

pET28b-pfDHODH₃₈₄₋₄₁₃ C276F was also expressed in BL21-DE3 *E. coli* phage-resistant cells for protein crystallization with the following modifications. Kanamycin (50 µg/ml) replaced ampicillin and pelleted cells were resuspended in lysis buffer B (100 mM HEPES pH 8.0, 150 mM NaCl, 10% glycerol, 10 mM imidazole, and 0.05% THESIT detergent (Fluka)) as previously described.^{22, 26} Cells were lysed as described above and purified by HisTrap HP column preequilibrated with lysis buffer. PfDHODH₃₈₄₋₄₁₃ C276F was eluted from the column using a linear gradient from 20 – 350 mM imidazole. Fractions containing pfDHODH₃₈₄₋₄₁₃ C276F were pooled, concentrated with Amicon Ultra concentrator (Millipore) and then purified by gel filtration column chromatography on a HiLoad 16/60 superdex 200 column (GE Healthcare) preequilibrated with crystallization buffer (20 mM HEPES, pH 7.8, 20 mM NaCl, 1 mM N,N-Dimethyldodecylamine N-oxide (LDAO, Fluka), 5% glycerol, 10 mM DTT). Fractions containing PfDHODH₃₈₄₋₄₁₃ C276F were pooled and concentrated to 28 mg/ml. Protein concentration was determined by UV280 using a molar extinction coefficient of 30590 M⁻¹cm⁻¹, as calculated by <https://web.expasy.org/protparam/>.

Enzyme Kinetic Analysis.

Kinetic enzyme assays were performed using either a direct assay that monitors the production of orotic acid at 296 nm or by coupling the reaction to the reduction of 2,6-dichloroindophenol (DCIP) at 25°C as described.¹⁷ For the direct assay, conditions were as follows. Assay buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100 reduced) supplemented with 50 mM glucose was mixed with DHODH substrates, 0.1 mg/ml of glucose oxidase and 0.02 mg/ml of catalase, followed by incubation for 5 min at room temperature prior to initiating the assay by adding enzyme.¹⁷ The oxidase/catalase system was included to eliminate non-CoQ_D-dependent background oxygen activity. To determine the K_m for CoQ_D, the direct assay was performed using 500 µM L-DHO over a range of CoQ_D (0–150 µM) in the presence of PfDHODH mutant (5–20 nM). To determine the K_m for L-DHO, the DCIP assay was performed using 150 µM CoQ_D over a range of L-DHO (0–500 µM) in the presence of the PfDHODH mutant (5–20 nM). Kinetic parameters (k_{cat} and K_m) were determined by fitting the rate versus substrate concentration data to the Michaelis-Menten equation using GraphPad Prism. The 50% inhibitory concentration (IC₅₀) for inhibition of the WT and mutant enzymes by DSM265 was also determined using the direct assay using a 3-fold serial dilution of inhibitor (0–100 µM) in the presence of 20 µM CoQ_D, 200 µM L-DHO and 5–20 nM enzyme. To determine the IC₅₀, data were fitted to log (inhibitor) vs. response equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{LogIC}_{50})})$.

PfC276F–DSM1 Crystallization, data collection and structure refinement.

Preliminary crystallization conditions were found using the random crystallization screen *Cryos* suite (Hampton Research) and conditions were then refined by varying the pH and precipitant concentration by hanging drop vapor diffusion at 20°C. The crystallization drop was mixed with an equal volume of reservoir solution and PfDHODH₃₈₄₋₄₁₃ C276F (28 mg/ml) pre-equilibrated with 1 mM DSM1 (0.1 M stock solution in DMSO) and 1 mM dihydroorotate (DHO, 0.1 M stock solution in DMSO). Crystals of the PfDHODH₃₈₄₋₄₁₃ C276F-DSM1 complex grew from 0.06 M ammonium sulfate, 0.1 M sodium acetate, pH

4.4, 14% PEG4000 (w/v), 24% glycerol (v/v), and 10 mM DTT. Crystals typically grew in one week.

Diffraction data were collected at 100K on beamline 19ID at Advanced Photon Source (APS) using an ADSC Q315 detector. The crystal of *PfDHODH*₃₈₄₋₄₁₃ C276F-DSM1 diffracted to 2.15 Å and displays symmetry consistent with the space group P6₄ with the cell dimension of a=b=85.5 Å, c=138.9 Å (Table 3). The structure contains only one molecule of *PfDHODH* in the asymmetric unit. Diffraction data were integrated and intensities were scaled with the HKL2000 package.³⁶

Crystallographic phases for *PfDHODH*₃₈₄₋₄₁₃ C276F-DSM1 were solved by molecular replacement with Phaser³⁷ using the previously reported structure of *PfDHODH*₃₈₄₋₄₁₃ bound to DSM1 (PDB ID 3I65²²) as a search model. Structures were rebuilt with COOT³⁸ and refined in PHENIX³⁹ to R_{work}/R_{free} of 0.178/0.205 respectively. Data processing and structure refinement statistics are shown in Table 3. The structure was displayed with PyMOL Molecular Graphics System (Version 1.8, Schrödinger). Structural alignments were performed in PyMOL using the align command. The coordinates have been submitted to the protein database (PDB ID 6E0B).

CRISPR/Cas9 editing to generate Dd2 parasites expressing C276F and C276Y mutant *PfDHODH*.

Cloning.—Three CRISPR/Cas9 guide RNA (gRNA) sequences were designed to edit the *Pfhdh* locus in Dd2 parasites. Guides 1 and 3 were designed using the online guide design tool ECRISP (Table S2).⁴⁰ Guide 2 was designed by manually scanning the *Pfhdh* gene for potential PAM sites, and was selected due to its proximity to amino acid position 276 (Table S2). These guides were expressed under the control of a 0.5 kb U6 snRNA promoter in a pDC2 based plasmid backbone.⁴¹ This plasmid also expresses a codon-optimized Cas9 endonuclease driven from the calmodulin 5' UTR, and a human dihydrofolate reductase (*hdhfr*) gene that confers resistance to WR99210 (Fig. 5A). A 1.6 kb region of the *Pfhdh* gene, which served as the homology donor repair template, was also cloned into this plasmid to yield the plasmid, pDC2-cam-coCas9-U6(gRNA)-donor-*hdhfr* (Fig. 5A). We used site-directed mutagenesis and introduced three sets of unique silent binding-site mutations (BSmut) for each guide, yielding three Cas9 BSmut control plasmids (Table S2). These mutations shield the plasmid from self-cleavage by the Cas9 endonuclease. We performed further site-directed mutagenesis on the three Cas9 BSmut control plasmids to introduce the C276F or C276Y mutations, yielding a total of six Cas9 editing plasmids.

Parasite transfections.—Purified plasmid DNA (50 µg) was electroporated into 10⁸ Dd2 parasites as previously described.⁴² Transfections with the three Cas9 Bsmut control plasmids were selected with 2.5 nM WR99210. Transfections with the six C276Y/F-editing plasmids received an additional pulse of DSM265 at 5× the Dd2 IC₅₀ for 6 days in addition to 2.5 nM WR99210. This short-term pulse helps select positively edited parasites in a mixed parasite population. Based on prior work, this electroporation would yield ~400 parasites,⁴³ which is multiple orders of magnitude below the minimum inoculum of resistance for the emergence of DSM265-resistant parasites (10⁵ or higher).¹² Earlier whole-

genome sequence analyses showed that gene editing, via CRISPR/Cas9 or the related method of zinc-finger nucleases, yields no off-target activity in *P. falciparum*.^{41, 44} Those results were consistent with *Plasmodium* lacking non-homologous end joining, forcing it to depend entirely on homology-directed recombination for DNA repair.⁴⁵ From the *Pfdhodh* plasmid electroporations, microscopically viable parasites were observed after 6–7 weeks, and these were screened for gene editing via blood PCR amplification and sequencing. Editing events for C276F, C276Y and the BSmut control parasite lines were observed with the Guide-2 plasmids. These bulk cultures were cloned via limiting dilution and were assessed for *in vitro* drug susceptibility to DSM265. All edited parasites harbored the BS mutations, indicating that all were a result of gene editing and were not spontaneous mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Structures of *P*/DHODH triazolopyrimidine based inhibitors. DSM1 was the initial hit identified by high-throughput screening.¹⁸ DSM265 is a clinical candidate¹² identified by subsequent lead optimization.

Fig. 2. Effect of *PfDHODH* point mutations on (A) *P. falciparum* efficacy and (B) on binding to the enzyme target. Data show the average EC₅₀ (*P. falciparum* whole cell assays) or IC₅₀ (50% enzyme inhibitor concentration) for independent experiments as described in Tables 1 and 2. Error bars represent the standard deviation of the mean.

Fig. 3. X-ray structure analysis of C276F *PfDHODH*.

This figure shows the structural alignment of C276F *PfDHODH* bound to DSM1 (pink) with WT *PfDHODH* bound to DSM265 (pdb 4rx0)¹² (tan). Select residues in the 4 Å inhibitor shell are shown. All residues that were found mutated in this study are shown. Dotted black lines show the hypothetical distance between I263 in the C276F structure with DSM265, showing a steric clash. Only side chains of the residues are shown. The bound cofactor FMN (flavin mononucleotide 5'-phosphate) and the product Oro (orotate) are also displayed.

		181	185	
<i>P. falciparum</i>	NPEFFLYDIFLKFKLYIDG	ICHDLFLLLGKYNILPYDTSNDSIYACTNIKHLDFINPF	221	
<i>P. vivax</i>	DPEFFLYDVFLKMLLKYYVDG	TCHDLFLLMGKYKLLPYDTGKDNIYSCSEIKGLNFINPF	223	
<i>P. cynomolgi</i>	DPEFFLYDVFLKMLLKYYVDG	TCHDLFLLMGKYSLLPYDTSKDSIYSCSEIKGLKFMNPF	45	
<i>P. berghei</i>	NPEFFMYDVFLDFCLNYVDS	VCHDLFLLLGKYGLLPYDTSNDSVYATSDIKNLFINPF	96	
HUMAN	GDERFYAEHLMP	TLQGLLDPSAHLRAVRFTSLGGLPRARFQSDMLEVRVLGHKFRNPV	91	
DOG	GDEHFYAEHLMP	QRLLDPSAHLRAVRVTSGLLPRATFQESDMLEVRVLGHRFRNP	110	
RAT	GDDHFYAEYLMPL	QLRLDPSAHLRAVRVTSGLLPRATFQSDMLEVVKVLGHKFRNPV	62	
MOUSE	GDDHFYAEYLMPL	QRLDPSAHLRAVRVISLGLLPRATFQDSNMLEVRVLGHKFRNPV	91	
		227	237	240
<i>P. falciparum</i>	GVAAGFDKNGVCID	SILKLGFSFIEIGTITPRGQTGNAPRIF	265	276
<i>P. vivax</i>	GVAAGFDKNGVCID	GILKLGFSFIEIGTITPKAQKGNRPRI	265	276
<i>P. cynomolgi</i>	GVAAGFDKNGVCID	GILKLGFSFIEVGTITPKAQKGNPKRI	265	276
<i>P. berghei</i>	GVAAGFDKNGICID	SILKLGFSFIEIGTITPKPKQGNKPRIF	265	276
HUMAN	GIAAGFDKHGEAVD	GLYKMGFGFVEIGSVTPKPQEGNPRPRV	265	276
DOG	GIAAGFDKHGEAVD	GLYKMGFGFVEIGSVTPKPQEGNPRPRV	265	276
RAT	GIAAGFDKNGEAVD	GLYKLGFGFVEVGSVTPKPQEGNPRPRV	265	276
MOUSE	GIAAGFDKHGEAVD	GLYKLGFGFVEVGSVTPKPQEGNPRPRV	265	276
		531	536	
<i>P. falciparum</i>	AGASVCQLYSC	VFNGMKSAVQIKRELNHLLYQRGYYNLKEAIGRKHSKS	569	
<i>P. vivax</i>	AGASVCQLYSC	VFNGMKAARVRIKRELDHLLYQRGYYKLGDVAGRAHRRRA	572	
<i>P. cynomolgi</i>	AGASVCQLYSC	VFNGMKAARVRIKRELDHLLYQRGYYKLEDAIGKAHRRRG	385	
<i>P. berghei</i>	AGASVCQLYSC	VFNGVKSATQIKREFNNALYQKGYNNLREAIGKKHNSAKSLKV	418	
HUMAN	AGASLVQLYTAL	TFWGPVVGKVKRELEALLKEQGFVVDAIGADHRR	395	
DOG	AGASLVQLYTAL	TYRGPVVGMIKRELEALLKEQGFTRITDAIGADHRR	414	
RAT	AGASLVQLYTAL	IFLGPVVRVKRELEALLKERGFVVDAIGADHRR	366	
MOUSE	AGASLVQLYTAL	TFLGPVVARVKRELEALLKERGFNTVDAIGVDHRR	395	

Fig. 4. Partial alignment of *Plasmodium* and mammalian DHODH sequences.

Numbering is based on the *Pf*DHODH sequence. Highlighted residues were identified in the *P. falciparum* DSM265-resistant clones. Residues highlighted in yellow are variable and residues highlighted in green are conserved. Sequences were obtained from PlasmoDB or the NCBI protein data-base: *P. falciparum* (PF3D7_0603300), *P. vivax* (PVX_113330), *P. cynomolgi* (PCYB_115310), *P. berghei* (PBANKA_010210), Human (NP_001352.2), dog (XP_853399.2), rat (NP_001008553.1) and mouse (NP_064430.1). The sequence alignment was generated using the web server <http://www.ebi.ac.uk> and CLUSTAL O(1.2.1) multiple sequence alignment program. *Pf*DHODH Residues 282–519 are not shown.

Fig. 5. Generation and analysis of CRISPR/Cas9 *P. falciparum* Dd2 lines containing the *PfDHODH* C276F and C276Y mutant alleles.

(A) Schematic of the CRISPR/Cas9 editing strategy of the *Pfhdh* locus. (B) DSM265 dose response curves for growth inhibition of wild-type and mutant parasite lines. EC₅₀ values were determined for 5 to 12 independent assays performed in duplicate (see Table S3) where error represents the standard deviation of the mean. Dose response data were fitted to the inhibitor vs response – variable slope (four parameters) equation to determine EC₅₀ in GraphPad Prism. (C) Comparative analysis of DSM265 EC₅₀ (μ M) on wild-type and CRISPR/Cas9-edited Dd2 lines. The EC₅₀ means $\pm$ standard deviation calculated from the independent dose response curves in (B) are plotted where: Dd2 parent (0.0048 ± 0.00086 μ M), Dd2 BSmut control (0.0051 ± 0.00031 μ M), Dd2^{Dhodh} C276F CL1 (0.36 ± 0.046 μ M), Dd2^{Dhodh} C276F CL2 (0.39 ± 0.23 μ M), Dd2^{Dhodh} C276Y CL1 (0.12 ± 0.026 μ M), Dd2^{Dhodh} C276Y CL2 (0.12 ± 0.024 μ M). *** $p < 0.0005$.

Table 1.Analysis of DSM265-resistant *P. falciparum* Dd2 clones.

Clonal line	DSM265 selection concentration (μM)	DSM265 *EC ₅₀ μM (<i>n</i>)	Fold change	Amino acid mutation (DNA mutation)	Gene copy number
Dd2 WT	--	0.0046 $\pm$ 0.0011 (10)	1	na	1
Dd2 R10C1B	0.023	0.15 $\pm$ 0.051 (5)	33	G181C (G541T)	1.1
Dd2 RIAC1B	0.060	0.17 $\pm$ 0.055 (8)	37	C276F (G827T)	1.1
Dd2 R1BC1A	0.036	0.11 $\pm$ 0.017 (5)	24	L531F (G1593T)	1
Dd2 R2B	0.036	0.15 $\pm$ 0.073 (4)	33	R265G (A793G)	0.9
Dd2 R3B	0.036	0.071 $\pm$ 0.013 (3)	15	E182D (A546T)	1

Selections of DSM265-resistant parasites were performed with an inoculum of 2×10^9 parasites, with the exception of the R10C1B clone (2×10^6) and were previously described.¹² Data sets were collected in triplicate for each concentration in the dose response curve and data were fitted in GraphPad Prism to determine the EC₅₀ using the LogI versus response equation (4 parameter). Reported data represent the average and standard deviation for multiple such experiments where the number of independent replicates (*n*) are shown in parenthesis. Statistical significance was evaluated using an unpaired two-tailed *t*-test to compare the average values for mutant data versus the Dd2 wild-type control.

* *p* values of < 0.0001 were obtained in all cases. EC₅₀ values on atovaquone and artemisinin were comparable to the parent Dd2 strain and are reported in Table S1.

Table 2.Enzyme kinetic analysis of recombinant mutant *P/DHODH*

Mutant	k_{cat} CoQD s^{-1} (<i>n</i>)	K_m CoQD μM (<i>n</i>)	k_{cat}/K_m CoQD $\mu M^{-1}s^{-1}$	K_m DHO μM (<i>n</i>)	DSM265 IC ₅₀ μM (<i>n</i>)	DSM265 IC ₅₀ Fold change (<i>p</i> value)
WT	15±3.4 (4)	34±11 (4)	0.44±0.17	63±5.6 (4)	0.019±0.012 (6)	na
G181C	6.0±1.0 (3)	18±2.5 (3)	0.33±0.072	29±8.4 (3)	0.34±0.21 (4)	18 (0.0056)
C276Y	65±19 (3)	130±34* (3)	0.50±0.20	67±30 (4)	0.15±0.038 (3)	7.9 (<0.0001)
C276F	13±7.9 (3)	38±8.5 (3)	0.34±0.22	54±14 (4)	0.15±0.10 (4)	7.9 (0.015)
L531F	22±8.7 (3)	35±8.7 (3)	0.63±0.29	19±2.2 (4)	0.043±0.017 (3)	2.3 (0.038)
R265G	47±24 (3)	130±72* (3)	0.36±0.27	25±1.7 (4)	0.63±0.056 (3)	33 (<0.0001)
E182D	12±6.3 (3)	18±5.1 (3)	0.66±0.40	140±31 (4)	0.15±0.11 (4)	7.9 (0.011)

Data sets were collected in duplicate (substrate data) or triplicate (inhibitor data) for each concentration in the dose response curve. CoQD K_m and k_{cat} data were collected with the direct assay, as were DSM265 IC₅₀ data. The K_m for DHO was determined using the DCIP assay. Data were fit to the Michaelis-Menten equation in GraphPad Prism to determine kinetic parameters (k_{cat} , K_m and k_{cat}/K_m) or to the LogI or I versus response equations (3 or 4 parameter) to determine (IC₅₀). Reported data represent the average and standard deviation for multiple such independent experiments where the number of independent replicates (*n*) are shown in parenthesis. Statistical significance was evaluated using an unpaired two-tailed *t*-test to compare the average values for mutant data versus the WT control, where *p* values < 0.05 are considered significant. There were no statistically significant changes in k_{cat}/K_m or k_{cat} for any of the mutants.

* *p* values for K_m changes between WT and mutant were significant (<0.05) in the following cases: for CoQ: C276Y (*p* = 0.0029) and for R265G (*p* = 0.042), and for DHO: L531F (*p* < 0.0001), G181C (*p* = 0.0013), R265G (*p* < 0.0001) and E182D (*p* = 0.0029).

Table 3.*P*ADHODH₃₈₄₋₄₁₃C276F-DSM1 X-ray diffraction data and refinement statistics

Data collection	
PDB ID code	6E0B
Space group	P6 ₄
Cell constants a, b, c (Å)	85.52, 85.52, 138.88
Wavelength (Å)	0.97918
Resolution range (Å)	50.0 – 2.10 (2.14 – 2.10)
Unique reflections	33,666 (1,679)
Multiplicity	11.0 (8.3)
Data completeness (%)	100 (100)
$R_{merge}(\%)^a$	5.0 (187)
$R_{pim}(\%)^b$	1.6 (67.9)
I/σ(I)	46.3 (1.2)
Wilson B-value (Å ²)	22.6
Refinement statistics	
Resolution range (Å)	42.77 – 2.10 (2.17 – 2.10)
No. of reflections R_{work}/R_{free}	28,985/1,462 (535/27)
Data completeness (%)	86.3 (16.7)
Atoms (non-H protein/cofactors/inhibitor/solvent)	2977/42/21/113
$R_{work}/R_{free}(\%)$	17.7 (25.5)/20.5 (30.7)
R.m.s.d. bond length (Å)	0.002
R.m.s.d. bond angle (°)	0.54
Mean B-value (Å ²) (protein/cofactors/inhibitor/solvent)	37.7/20.7/26.4/36.8
Ramachandran plot (%) (favored/additional/disallowed) ^c	97.9/2.1/0.0

Data for the outermost shell are given in parentheses.

^a $R_{merge} = 100 \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, where the outer sum (h) is over the unique reflections and the inner sum (i) is over the set of independent observations of each unique reflection.

^b $R_{pim} = 100 \sum_h \sum_i [1/(n_h - 1)]^{1/2} |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, where n_h is the number of observations of reflections **h** as defined by Evans⁴⁶

^cAs defined by the validation suite MolProbity⁴⁷