Structural Plasticity of Malaria Dihydroorotate Dehydrogenase Allows Selective Binding of Diverse Chemical Scaffolds

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Malaria remains a major global health burden and current drug therapies are compromised by resistance. Plasmodium falciparum dihydroorotate dehydrogenase (PfDHODH) was validated as a new drug target through the identification of potent and selective triazolopyrimidine-based DHODH inhibitors with anti-malarial activity in vivo. Here we report x-ray structure determination of PfDHODH bound to three inhibitors from this series, representing the first of the enzyme bound to malaria specific inhibitors. We demonstrate that conformational flexibility results in an unexpected binding mode identifying a new hydrophobic pocket on the enzyme. Importantly this plasticity allows PfDHODH to bind inhibitors from different chemical classes and to accommodate inhibitor modifications during lead optimization, increasing the value of PfDHODH as a drug target. A second discovery, based on small molecule crystallography, is that the triazolopyrimidines populate a resonance form that promotes charge separation. These intrinsic dipoles allow formation of energetically favorable H-bond interactions with the enzyme. The importance of delocalization to binding affinity was supported by site-directed mutagenesis and the demonstration that triazolopyrimidine analogs that lack this intrinsic dipole are inactive. Finally, the PfDHODH-triazolopyrimidine bound structures provide considerable new insight into species-selective inhibitor binding in this enzyme family. Together, these studies will directly impact efforts to exploit PfDHODH for the development of anti-malarial chemotherapy.

The human malaria parasite is endemic in 87 countries putting 2.5 billion people in the poorest nations of the tropics at risk for the disease (1, 2). Despite intensive efforts to control malaria through combination drug therapy and insect control programs, malaria remains one of the largest global health problems. The most severe form of the disease is caused by Plasmodium falciparum, which kills 1–2 million people yearly, primarily children and pregnant woman. Effective vaccines have not been developed, and chemotherapy remains the mainstay of both treatment and prevention of the disease. Unfortunately widespread drug resistance to almost every known anti-malarial agent has compromised the effectiveness of malaria control programs (3). The introduction of artemisinin combination chemotherapy has provided new treatment options to combat drug-resistant parasites (4). However, recent reports by the World Health Organization suggest that resistance to artemisinin is developing along the Thai-Cambodian border, underscoring the need for a continual pipeline of new drug development to combat this disease.

The malaria parasite relies exclusively on de novo pyrimidine biosynthesis to supply precursors for DNA and RNA biosynthesis (5, 6). In contrast, the human host cells contain the enzymatic machinery for both de novo pyrimidine biosynthesis and for salvage of preformed pyrimidine bases and nucleosides. The lack of a redundant mechanism to acquire pyrimidines in malaria has raised interest in this pathway as a potential source for new therapeutic targets. Dihydroorotate dehydrogenase (DHODH) is a flavin mononucleotide (FMN)-dependent mitochondrial enzyme that catalyzes the oxidation of dihydroorotate (DHO) to produce orotate, the fourth step in de novo pyrimidine biosynthesis. Situated at the intersection of two major metabolic pathways—pyrimidine and amino acid synthesis—DHODH is considered a potential drug target for the treatment of malaria and various other diseases (5). Several chemical classes of DHODH inhibitors have been identified, including triazolopyrimidines and triazolopyrimidine analogs. This work provided new insight into the species-selective inhibition of DHODH and increased the value of PfDHODH as a drug target. The most severe form of the disease is caused by Plasmodium falciparum, which kills 1–2 million people yearly, primarily children and pregnant woman. Effective vaccines have not been developed, and chemotherapy remains the mainstay of both treatment and prevention of the disease. Unfortunately widespread drug resistance to almost every known anti-malarial agent has compromised the effectiveness of malaria control programs (3). The introduction of artemisinin combination chemotherapy has provided new treatment options to combat drug-resistant parasites (4). However, recent reports by the World Health Organization suggest that resistance to artemisinin is developing along the Thai-Cambodian border, underscoring the need for a continual pipeline of new drug development to combat this disease.

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Novo pyrimidine biosynthesis (7, 8). Coenzyme Q (CoQ) is required to catalyze the reoxidation of the flavin cofactor, and recent genetic studies suggest that the main function of mitochondrial electron transport in the parasite is to supply CoQ for this reaction (9). These studies provide genetic evidence that PfDHODH is an essential enzyme to the malaria parasite. An inhibitor of human DHODH (hDHODH) (A77 1726 the active metabolite of leflunomide) is marketed for the treatment of rheumatoid arthritis, illustrating that DHODH is a druggable target (10, 11). Finally, biochemical (12, 13) and structural studies (14, 15) suggested that the identification of species-selective inhibitors against this target was feasible.

Our recent studies have since directly led to the validation of PfDHODH as a new target for the discovery of anti-malarials. We utilized a high throughput screen to identify several classes of potent and species selective inhibitors of PfDHODH (12, 16–18). These compounds are competitive with CoQ and inhibit the CoQ-dependent oxidation of FMN while not affecting the FMN-dependent oxidation of DHO. Site-directed mutagenesis data supported a model whereby the CoQ-binding site does not overlap with the inhibitor site, but instead inhibitors either block electron transfer between FMN and CoQ or stabilize a conformation that excludes CoQ binding (18, 19). Of the identified inhibitors, one promising series has emerged based on a triazolopyrimidine core structure (see Fig. 1 and Table 1). PfDHODH inhibitors in this class show potent nontoxic activity against P. falciparum in vitro, with excellent correlation observed between inhibition of PfDHODH and activity against the parasite (17, 18). We identified a metabolically stable derivative of this series (DSM74) that is able to suppress malaria in vivo (17). Interestingly, despite a vast number of ongoing attempts, PfDHODH represents one of only a few truly new targets for the development of anti-malarial agents since the discovery that atovoquone targets the cytochrome bc1 complex in the mitochondria (20). This has led to a substantial effort to target PfDHODH for drug discovery programs and to the identification of diverse scaffolds showing species-selective inhibition of the enzyme (17, 18, 21–24). The prior structure of PfDHODH complexed to A77 1726 (14), a hDHODH-specific inhibitor with poor affinity for PfDHODH (19), neither explains the ability of PfDHODH to bind the array of identified inhibitors nor provides an understanding of the developing SAR for the triazolopyrimidine-based inhibitor series.

Here we report the x-ray structures of PfDHODH bound to three triazolopyrimidine-based inhibitors with different sized substituents bound to the triazolopyrimidine core. In addition, we examined the small molecule x-ray structures of these inhibitors and compared them with the protein-bound ligand structures. Finally, the inhibitor-bound PfDHODH structures were compared with the structures of hDHODH bound to A77 1726 and to breguina, a potent 4-quinolinecarboxylic acid inhibitor of the human enzyme (25, 26). Together these studies explain both the high affinity binding and species selectivity of an important class of PfDHODH inhibitors, thus laying the foundation for future lead optimization programs for the present anti-malarial agents. More broadly, this study provides new, unexpected insight into why PfDHODH is a highly attractive drug target for a large set of diverse chemical entities with potential for sustainable, robust lead optimization programs.

EXPERIMENTAL PROCEDURES

Gene Cloning of PfDHODH—N-terminally truncated PfDHODH (amino acids 159–569) was PCR-amplified with primers 1 (5′-AAGGATCCGTTTTGAATCTTATAAC-CCG-3′) and 4 (5′-GGGTCGACTTCCATGTTACCGT-GAG-3′) from plasmid pRS7-pFDHODH encoding the codon optimized gene (provided by Jon Clardy) (14) and ligated into the pET28b (Novagen) expression vector at the BamHI/Sall sites. PfDHODH A384–413 was generated by PCR amplification of two fragments with compatible restriction sites that excluded the loop sequence. Fragment 1 (amino acids 159–383) containing 5′-BamHI and 3′-EcoRI was generated using primers 1 and 2 (5′-GGAATTCGTCATTCATAATGTT-TATTTT-3′) and fragment 2 (amino acids 414–569) containing 5′-EcoRI and 3′-Sall was PCR-amplified with primers 3 (5′-GGGATCCCTGTTAATTATCAGGAA-3′) and 4. Both PCR products were subcloned separately into pCR-Blunt II-TOPO vector (Invitrogen). The resulting clones were used to generate the final expression clone containing loop-truncated PfDHODH A384–413 in pET28b in frame with an N-terminal His tag.

Protein Expression and Purification—Escherichia coli BL21 phage-resistant cells (Novagen) were used for the expression of pET28b wild-type PfDHODH and PfDHODH A384–413 constructs using a modification of methods described previously (19). Proteins were expressed in Terrific Broth medium containing kanamycin (50 μg/ml). The cells were grown to 0.8 A600 at 37 °C, 0.2 mM isopropyl-β-D-thiogalactoside was added to induce protein expression, and the cells were grown overnight at 16 °C. The cells were pelleted by centrifugation (4000g) and resuspended in lysis buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM glycerol, and 0.05% THESIT detergent (Fluka)), containing protease-inhibitor mixture for His tag protein (Sigma). The cells were lysed by three passes through an EmulsiFlex-C5 high pressure homogenizer (Avestin Inc.), the lysate was clarified by centrifugation (20,000g), and the resulting supernatant was applied to a HisTrap HP column (GE Healthcare) precharged with Ni2+. The column was eluted with buffer containing kanamycin (20,000 × g), and the resulting supernatant was applied to a HiTrap HP column (GE Healthcare) precharged with Ni2+. The column was sequentially washed with lysis buffer and lysis buffer containing 20 mM imidazole. PfDHODHs were eluted from the column using a linear gradient from 20 to 400 mM imidazole. Fractions containing PfDHODH were pooled, concentrated with Amicon Ultra concentrator (Millipore), and then purified by gel filtration chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with crystallization buffer (10 mM HEPES, pH 7.8, 100 mM NaCl, 1 mM N,N-diethylodecylamine-N-oxide (Fluka), 5% glycerol, 10 mM dithiothreitol). Fractions containing PfDHODH were pooled and concentrated to 20 mg/ml. The construction and purification of the H185A, F188A, F227A, and R265A mutant PfDHODH enzymes were described previously (12, 19).

Enzyme Kinetic Analysis—Steady-state kinetic analysis was performed as described previously (12, 19). To determine the
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The reactions were performed in assay buffer (100 mm HEPES, pH 8.0, 150 mm NaCl, 10% glycerol, 0.1% Triton) at 20 °C for a range of CoQD (0.025–0.15 mm) and DHO concentrations (0.01–0.5 mm) with PfDHODH (2 nm). Inhibitor kinetics for both wild-type (10 nm) and mutant (10 nm) PfDHODH were followed using the indirect assay that couples the oxidation of DHO (0.2 mM) in the presence of CoQD (20 μM) to the reduction of 2,6-dichloroindophenol (60 μM) at 600 nm (ε = 18.8 mm⁻¹ cm⁻¹). The data were fitted to the Michaelis-Menten equation to determine the steady-state kinetic parameters or to the following equation to determine the IC₅₀ values using Graph Pad Prism (Graph Pad), 

\[ v_i = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(\log(V_i) - \log(\text{IC}_{50})})} \]

where, \( v_i \) is the initial velocity (s⁻¹), and Top and Bottom represent the plateaus in velocity units.

**Crystallographic and Data Collection of PfDHODH**

**PfDHODH-DSM1 and DSM2 Bound to Inhibitors**—Random crystallization screen Cryo suite (Nossal) and detergent screen kits (Hampton Research) were utilized to determine preliminary crystallization conditions. Subsequent refinement of pH, precipitant, detergent, and protein concentrations was then done to find optimal conditions. N,N-Dimethyldodecylamine N-oxide (1 mm) was found to improve crystalization, and it was subsequently added during the final gel filtration purification step. The crystals of PfDHODH-H₁₁₀₀₅, with the cell dimension of \( a = b = 85.9, c = 138.4; \) PfDHODH-DSM2 diff acted to 2.4 Å with space group P6₁, and cell dimension of \( a = b = 85.9, c = 138.7; \) and PfDHODH-DSM74 diff acted to 2.7 Å with space group P6₁, and cell dimension of \( a = b = 85.4, c = 138.6 \) (supplemental Table S2). All three structures contain residues Ser₁⁶⁰–Ser₅⁶⁷, with the exception that density was not observed for a loop formed by residues 348–355, 124 water molecules, and a bound DHO detergent molecule. The final DSM2- and DSM74-bound PfDHODH structures contain all residues between Glu¹⁹³–His⁵⁰⁶ (PfDHODH-DSM2) and Phe¹⁶¹–His⁵⁰⁶ (PfDHODH-DSM74), plus 63 and 38 water molecules, respectively. No density was observed for the detergent molecule in these structures. In the DSM1-, DSM2-, and DSM74-bound structures the shortened surface loop at position 384 is observed in its entirety.

**Molecular Modeling**—The structures were displayed using the graphics program PyMol (26). The PfDHODH-DSM1 structure was superimposed with the PfDHODH-DSM2, PfDHODH-DSM74, PfDHODH-A₇₇ (Protein Data Bank code 1tv5), hDHODH-A₇₇ (Protein Data Bank code 1D3H) (15), and hDHODH-bre (Protein Data Bank code 1D3G) (15) structures by aligning only backbone atoms of the βα domain in LSUQAB (33). For the alignment between PfDHODH-DSM1 and hDHODH, the following sequences were superimposed: P'₁²⁷⁻⁻²³² to h₈₈⁻⁻¹⁰₃; P'₁²⁷⁻⁻²³² to h₉₁₄⁻⁻¹₄₉; P'₃₃₇⁻⁻₄₃₄ to h₉₂⁰⁻⁻²₁₃; P'₃₄₆⁻⁻₄₆₀ to h₉₆₂⁻⁻₂₈₆; P'₄₇₃⁻⁻₄₉₄ to h₃₀₁⁻⁻₃₂₂; P₅₀₁⁻⁻₅₂₉ to h₃₂₉⁻⁻₃₅₇; and P₅₄₁⁻⁻₅₅₂ to h₃₆₉⁻⁻₃₈₀. The same PfDHODH residues were used to superimpose the various PfDHODH structures. RMSD values were calculated for the superimposed structures based on the Cα positions using LSQM (32, 34). Moleman2 (35) was used to manipulate the Protein Data Bank files before the analysis (Software was obtained from the Uppsala Software Factory).

**Small Molecule X-ray Structure Determination**—Crystallization of DSM1 from CH₂Cl₂/CH₃OH was described previously (18). DSM15, DSM16, and DSM74 were crystallized similarly to CH₂Cl₂. Single crystals were mounted on a glass capillary with oil. The data were collected at −143 °C with a Nonius Kappa CCD FR590 single crystal x-ray diffractometer. The crystal-to-detector distance was 30 mm. Other data collection parameters are summarized in supplemental Table S3. The data were integrated and scaled using hkl-SCALEPACK (27). Solution by direct methods (SIR97) (36) produced a complete heavy atom phasing model (supplemental Table S4). All of the hydrogen atoms were located using a riding model with the exception of H₁. All of the non-hydrogen atoms were refined anisotropically by full matrix least squares utilizing SHELXL97 (37). Scattering factors are from Waasmaier and Kirfel (38), and the absolute structure assignment for DSM1 was established via anomalous scattering. The Flack enantiopole parameter (39) is −0.06 ± 0.11. ORTEP drawings (40) are shown in supplemental Fig. S4.

The crystals of DSM15 were twinned such that two lattices of very similar intensities were apparent in the diffraction pattern. One lattice was indexed. In a first refinement cycle, intensities of reflections of type {0 k l} (10% of total data) appeared systematically larger than those calculated. Excluding {0 k l} reflections from the refinement allowed a useful structure to be determined. The ratio between the calculated and measured intensities of the excluded data was close to 2, consistent with a total overlap of two data sets. Such twinning can arise from 2-fold twinning along [1 0 0] or a mirror on the [0 k l] plane. No additional twin law was found for the reduced data set, indicating that the twinning did not create any detectable intensity.
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![Inhibitors of dihydroorotate dehydrogenase. A, selective inhibitors of PfDHODH. DSM1, DSM2, and DSM74. B, selective inhibitors of human DHODH, A77 1726, and brequinar.](image)

Overlaps for other peaks. Thus the excluded intensity data were corrected for the factor 2 and merged with the other data for the final structural refinement.

For DSM74 some of the intermolecular fluoride-fluoride distances were too short. This problem arises from disorder of the CF₃ group. CF₃ groups are very often found to rotate or be arranged in two alternating geometries. This is the case in this structure where two conformations of the CF₃ (atoms F1–3 and F4–6) were refined at half-site occupancies including the carboxyl group. CF₃ groups are very often found to rotate or be arranged in two alternating geometries. This is the case in this structure where two conformations of the CF₃ (atoms F1–3 and F4–6) were refined at half-site occupancies including the carboxyl group.

RESULTS

X-ray Structure Determination of PfDHODH Bound to Triazolopyrimidine Analogs—Three triazolopyrimidine analogs containing naphthyl (DSM1), anthracenyl (DSM2), and phenyl-trifluoromethyl (DSM74) substituents, which span a range of inhibitor potency (0.05–0.3 μM), were chosen for crystallographic analysis (Fig. 1 and Table 1). Proteolysis of a P. falciparum-specific surface loop, which is not present in the enzymes from human or other Plasmodium species (residues 384–413; Fig. 2), led to difficulties obtaining diffraction quality crystals with these inhibitors. To improve crystallization we generated a PfDHODH construct lacking amino acid residues 384–413 (PfDHODHΔ384–413). This region of the structure was missing or disordered in the PfDHODH structure described previously (14). Steady-state kinetic analysis of PfDHODHΔ384–413 demonstrated...
that the catalytic efficiency and inhibitor binding properties of the loop-minus protein were similar to the wild-type enzyme (supplemental Table S1). *P. falciparum* DHODH was co-crystallized with DSM1, DSM2, or DSM74 in the presence of substrate DHO, and 2.0, 2.4, and 2.7 Å resolution data sets were collected, respectively. The three structures were solved by molecular replacement and refined to R_{free} of 23.1, 22.5, and 23.6 and R_{free} of 24.8, 26.7, and 27.6, respectively (supplemental Table S2).

### TABLE 1

Inhibition kinetics of wild-type and mutant DHODH

The error represents the standard error of the mean for n = 4–5. The fold change is shown in parentheses. The IC_{50} values for A77 1726 against *Pf* DHODH and *hDHODH* are 180 μM (19) and 0.3–1 μM (54–56), respectively. Brequinar binds *hDHODH* with an IC_{50} of 0.006–0.01 μM (45).

| Inhibitor enzyme | DSM1 | DSM2 | DSM74 |
|------------------|------|------|-------|
| *Pf* DHODH       | 0.047^{a} | 0.056^{a} | 0.28^{b} |
| *hDHODH*         | >100^{a} | >100^{a} | >100^{a} |
| *Pf* DHODH-H185A | 1.3 ± 0.17 (28) | 1.3 ± 0.17 (23) | 25 ± 9.0 (90) |
| *Pf* DHODH-R227A | 2.1 ± 0.70 (45) | 2.4 ± 1.1 (43) | 23 ± 6.7 (82) |
| *Pf* DHODH-F188A | 2.1 ± 0.08 (28) | 4.4 ± 1.1 (79) | 6.3 ± 1.1 (23) |
| *Pf* DHODH-F188A | 2.1 ± 1.4 (45) | 0.21 ± 0.04 (4) | 1.4 ± 0.35 (7) |

^{a} Data taken from Ref. 18. ^{b} Data taken from Ref. 17.

The three-dimensional fold of *Pf* DHODH in all three inhibitor bound complexes is similar to the previously reported structures of *Pf* DHODH bound to A77 1726 (14) and of human DHODH (hDHODH) (15). The enzyme consists of a short N-terminal helical domain (residues 163–194) followed by a classic βαβ barrel domain that begins with β-strand 3 (Fig. 2 and 3A). The electron density maps of the active sites of all three structures showed strong, interpretable density for the bound inhibitors (DSM1, DSM2, or DSM74) (supplemental Fig. S1).

**Triazolopyrimidine-binding Site**—The inhibitor-binding site is formed adjacent to the FMN site between the two N-terminal helices (α1 and α2), the top of helix α3 in the βα barrel, the 3_{10}-helix α11, and strand β5 (Fig. 3A). The naphthyl of DSM1 interacts with α3, whereas the triazolopyrimidine group lies against α11. DSM1, DSM2, and DSM74 bind in the same site. The inhibitors are oriented such that the C-5 position of the triazolopyrimidine ring is the closest atom to FMN at a distance of 6 Å (Fig. 3). The inhibitors are bound in an extended conformation with the naphthyl (DSM1), anthracenyl (DSM2), or phenyl-trifluoromethyl (DSM74) groups oriented away from the triazolopyrimidine core and from FMN. The triazolopyrimidine ring in all three structures binds to a largely hydrophobic pocket formed by Val^{332}, Leu^{172}, Leu^{176}, Cys^{184}, and Gly^{181} (Fig. 3B and supplemental Fig. S2) that also contains two residues that form the only nonhydrophobic contacts in this pocket. These nonhydrophobic contacts include ion pair H-bonds between His^{185} and the bridging nitrogen N-1 and between Arg^{265} and the pyridine nitrogen N-5 (Fig. 4, A and B). We have defined this pocket as the H-bond pocket (Fig. 4C). The naphthyl (DSM1), anthracenyl (DSM2), or phenyl-trifluoromethyl (DSM74) groups bind in a completely hydrophobic pocket formed by residues Leu^{172}, Leu^{176}, Met^{265}, and Phe^{227}, and Phe^{188} (Fig. 3B and supplemental Fig. S2), which we will define as the Pf-naphthyl pocket (Fig. 4C). An edge-to-face stacking interaction between Phe^{227}, the aromatic group bound in the Pf-naphthyl pocket, and Phe^{188}, is present (Fig. 4A), suggesting that these interactions likely contribute to the potent binding of the inhibitor series. These interactions involve the inhibitor in an extended aromatic stacking network that projects from FMN through Tyr^{528} to Phe^{227} on one face of the inhibitor and from Phe^{188} to Phe^{171} and beyond on the other side.

The van der Waal's surface of the DSM1-binding pocket shows that the inhibitor is buried in the interior...
of the protein (Fig. 4B). The naphthyl is bound snugly within a dead end hydrophobic pocket and the triazolopyrimidine ring, and the methyl side chain at C-6 also fit in close contact with the protein. A narrow channel leads from C-5 of the triazolopyrimidine ring toward FMN. An ordered water molecule (Wat15) in this channel is observed in all three structures forming an H-bond with N-3 of the inhibitor and with the hydroxyl of Tyr528, which in turn stacks against the FMN cofactor (Fig. 4A).

This channel then extends from the FMN to the protein surface providing the only access to solvent for the bound ligands (FMN, orotate, and inhibitor). However, this channel is too narrow to allow passage of either substrate or inhibitor, and thus significant structural movement must accompany ligand binding.

**Mutagenesis of Inhibitor-binding Pocket**—The contribution to binding affinity of residues involved in H-bond (His<sup>185</sup> and Arg<sup>265</sup>) or π-stacking interactions (Phe<sup>227</sup> and Phe<sup>188</sup>) (Figs. 3 and 4 and supplemental Fig. S2) were probed by site-directed mutagenesis. We previously published the effects of mutating these four residues to Ala and demonstrated that these mutations had only minimal effects on catalytic activity (k<sub>catalytic</sub> and K<sub>m</sub> remain within 2–4-fold of the wild-type enzyme) (19). In contrast, mutation of each of these residues to Ala increased the IC<sub>50</sub> for DSM1 by 30–50-fold, demonstrating that each residue contributes significant binding energy to the enzyme inhibitor interaction (Table 1). The relative contribution of the mutated residues differs for the three inhibitors described in this study. For DSM2, the contribution of His<sup>185</sup> and Arg<sup>265</sup> is similar to DSM1; however Phe<sup>188</sup> appears to play a reduced role in binding of this inhibitor. For DSM74, the H-bonds/ ion pairs between His<sup>185</sup> and Arg<sup>265</sup> and inhibitor contribute more energy to the binding interaction than Phe<sup>188</sup>. The IC<sub>50</sub> is increased by 80–90-fold for mutation of His<sup>185</sup> and Arg<sup>265</sup>, but only by 5-fold upon mutation of Phe<sup>188</sup>. We previously analyzed only the F227A and R265A mutant enzymes against DSM1 (18); the IC<sub>50</sub> reported for R265A was similar to the value reported here in Table 1. However, the previously measured IC<sub>50</sub> for F227A was 30-fold higher; solubility problems may have contributed to this elevated value.

**Small Molecule Structures of DSM Derivatives**—Complementary insight into the importance of N-1 of the protein (Fig. 4B). The naphthyl is bound snugly within a dead end hydrophobic pocket and the triazolopyrimidine ring, and the methyl side chain at C-6 also fit in close contact with the protein. A narrow channel leads from C-5 of the triazolopyrimidine ring toward FMN. An ordered water molecule (Wat15) in this channel is observed in all three structures forming an H-bond with N-3 of the inhibitor and with the hydroxyl of Tyr<sup>224</sup>, which in turn stacks against the FMN cofactor (Fig. 4A). This channel then extends from the FMN to the protein surface providing the only access to solvent for the bound ligands (FMN, orotate, and inhibitor). However, this channel is too narrow to allow passage of either substrate or inhibitor, and thus significant structural movement must accompany ligand binding.

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**Small Molecule Structures of DSM Derivatives**—Complementary insight into the importance of N-1
(41), respectively). Additionally, partial positive charge character of N-1 is further suggested by hydrogen bonding to a chloride ion in the DSM1 structure. These data suggest that when the bridging atom is N-1, resonance contributes to greater electron delocalization to N-5, intrinsically establishing long range dipoles in the inhibitor. In the protein, electron delocalization of the inhibitor is exploited by the H-bond with His185, which results in sufficient electron density on N-5 to allow formation of an ion pair with Arg265 (Figs. 4A and 5B). Such electron delocalization is more limited, or absent, for molecules containing O or S in the bridging position, providing an explanation for their inactivity as PfDHODH inhibitors. Thus the intrinsic dipole of the triazolopyrimidine ring is a key factor in the potency of this compound class toward PfDHODH.

Not all aspects of the small molecule conformation translate to what is observed on the protein. The plane of the naphthyl group in the protein-bound state is twisted by 48° relative to its unbound conformation (Fig. 5A). This conformational difference is necessary to position the naphthyl group of DSM1 to interact productively with the enzyme.

Plasticity to Accommodate Different Sized Triazolopyrimidine Inhibitors—Globally, the DSM1, DSM2, and DSM74-bound PfDHODH structures are very similar with RMSD for superimposition of the Cα atoms of 0.4 Å among them (Figs. 6A). The inhibitors occupy very similar positions in all three structures. The different size N-1 substituents are accommodated by conformational flexibility in the binding pocket. In both the PfDHODH-DSM2 and PfDHODH-DSM74 structures the triazolopyrimidine ring tilts slightly toward the bottom of the H-bond pocket relative to PfDHODH-DSM1. Leu176 reorients to accommodate this change. Further for PfDHODH-DSM74, this slight shift (0.2 Å) brings Ile263 into van der Waal’s contact with the C-5 position of the inhibitor. For PfDHODH-DSM2, the anthracenyl extends further into the binding site than naphthyl of DSM1. The larger aromatic group is accommodated by small rotational changes in Leu197 and Met536 that enlarge the Pf-naphthyl pocket. New contacts are also made with Leu240 and Cys233. The smaller phenyl-trifluoromethyl group of DSM74 does not completely fill the pocket and makes fewer van der Waal’s contacts. In particular Ile237 and Leu189 are no longer within the 4 Å van der Waal’s shell of DSM74. However, a new contact is made between the CF3 group and Leu240 in this structure. The reduced surface area of contact likely explains why DSM74 is 10-fold less potent than DSM1 or DSM2.
Structure of Malarial DHODH Bound to Potent Inhibitors

Conformational Flexibility of PfDHODH Accounts for Binding to Diverse Chemical Scaffolds—Comparison of the PfDHODH-DSM1 with PfDHODH-A77 structures shows that PfDHODH can present two alternative binding sites for interactions with different inhibitor classes. The PfDHODH-DSM1 and PfDHODH-A77 structures superimpose with a Cα RMSD of 1.03 Å, much larger than the 0.4 Å RMSD observed between the three triazolopyrimidine class inhibitors. Both inhibitors occupy the H-bond pocket such the triazolopyrimidine ring and the β-hydroxy enamide portion of A77 1726 overlap in this site (Figs. 4C and 6B). In contrast, the aromatic N-1 substituent in the triazolopyrimidines binds in an entirely different position than the aromatic N-1 substituent of brequinar binds the same site (H-bond pocket) as the triazolopyrimidine ring, forming a salt bridge between the carboxylate and hArg356 (equivalent to P. falciparum Arg265) interacting with N-5 of DSM1. This interaction is thought to play a major role in driving binding affinity (15, 42). In contrast, the biphenyl portion of brequinar binds to a distinct and different hydrophobic site (defined as the h_interstitial binding pocket) from the naphthyl-group of DSM1, which binds PfDHODH at the adjacent malaria-specific site (Pf\_naphthyl pocket). The h_interstitial hydrophobic pocket lies between the DSM1 Pf\_naphthyl pocket and the PfA77 phenyl pocket of A77 1726 when bound to PfDHODH (Fig. 4C).

These sites represent alternative pockets that are species-specific and are created by the different amino acid composition of the pockets (Fig. 7, A and B). The h_interstitial hydrophobic pocket that binds the brequinar biphenyl group is occluded in PfDHODH by the replacement of helix1 with the larger hAla59 and hPro364 in PfDHODH (defined as the Pf\_naphthyl hydrophobic pocket) from the naphthyl portion of A77 1726. Furthermore, in PfDHODH the naphthyl pocket is opened up relative to hDHODH by the replacement of hThr63 and hMet111 with hGly192 and hLeu240. Further the positions of hPhe227 and hLeu351 relative to the equivalent residues in hDHODH (hPhe98 and hLeu359) have swung away from the naphthyl position in PfDHODH-DSM1, enlarging the pocket relative to hDHODH. Globally the N-terminal helix (Ser160 to Gly200) relative to when bound to PfDHODH (Fig. 7A). This difference contributes to the opening of the h_interstitial hydrophobic pocket in PfDHODH.

A77 1726 is smaller than brequinar and does not extend as deeply into the h_interstitial hydrophobic pocket (Fig. 7C). Furthermore, the phenyl-trifluoromethyl group binds slightly closer to the Pf\_naphthyl pocket then brequinar. The β-hydroxy enamide portion of A77 1726 is flipped over in the hDHODH structure relative to when bound to PfDHODH. This changes the angle of projection of the phenyl-trifluoromethyl group into the hydrophobic pocket, allowing it to access the h_interstitial hydrophobic pocket instead of the Pf\_naphthyl pocket. Similar residues contribute to the differential binding as was observed for brequinar. Again the replacement of hAla59 with hPhe188 and hPro364 with...
**DISCUSSION**

Malaria remains one of the most significant global health problems because of wide spread drug resistance compromising current chemotherapies. One of the greatest challenges in developing new anti-malarial agents is the identification of novel targets that will allow the discovery of unexplored chemical scaffolds. Our discovery of the triazolopyrimidine-based inhibitors of *Pf*DHODH has led to the validation of *Pf*DHODH as a target for the development of new anti-malarials and to the identification of a novel chemical class with *in vivo* anti-malarial activity (17, 18). Here we report the x-ray structures of *Pf*DHODH bound to three triazolopyrimidine-based inhibitors. First, these data show that structural plasticity allows the triazolopyrimidine-based inhibitors to access an unexpected hydrophobic binding pocket that was not observed in previous structures. This structural flexibility allows the target to accommodate a range of inhibitors from different structural classes. A second unexpected insight was gained by comparison of the small molecule x-ray structures of the inhibitors with the protein-bound ligand structures. The intrinsic electronic configuration of the triazolopyrimidine ring favors charge delocalization from N-1 to N-5, providing significant insight into the emerging SAR of the triazolopyrimidine-based series. Finally, the variability in amino acid sequence between the human and malaria DHODH inhibitor-binding sites leads to species-selective inhibitor binding modes that explain the selectivity of the triazolopyrimidine-based inhibitors.

*Pf*Met^{536} closes off the phenyl-trifluoromethyl pocket of A77 1726 in *Pf*DHODH relative to the *h*DHODH structure.

**FIGURE 7.** Comparison of *Pf*DHODH-DSM1 to *h*DHODH inhibitor complexes. A, ribbon diagram of *Pf*DHODH-DSM1 (teal) aligned with *h*DHODH-bre (purple). Inhibitors are displayed as space filling balls. B, inhibitor-binding site alignment of *Pf*DHODH-DSM1 (teal) with *h*DHODH-bre (purple). C, inhibitor-binding site alignment of *Pf*DHODH-DSM1 (teal) with *h*DHODH-A77 (purple). Residue numbers for *h*DHODH are marked with a prime symbol, whereas *Pf*DHODH numbers are not. Inhibitors are displayed as ball and stick.
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naphthyl-functionality to insert into an extended edge-to-face stacking network that runs through the protein. Alternatively the inhibitors may selectively bind and stabilize a pre-existing conformation of the aromatic network that is part of the native state ensemble but that has not previously been observed. Aromatic edge-to-face stacking interactions provide additional binding energy beyond a typical van der Waal’s contact (52) as has been observed previously for potent binding of carboxypeptidase inhibitors (53). Our data support a role for these interactions in inhibitor binding to PfDHODH because mutation of residues in this network (Phe$^{227}$ and Phe$^{188}$) resulted in a significant reduction in binding affinity.

The intrinsic electronic properties of the triazolopyrimidine ring allow it to populate a resonance form that promotes charge separation, which is key to the formation of H-bond interactions with the enzyme. The importance of these ion pair interactions to inhibitor potency is supported both by the site-directed mutagenesis data and by the chemical modification of the inhibitor. When N-1 is replaced with atoms that are unable to delocalize electrons into the triazolopyrimidine ring (S or O), the $IC_{50}$ increases by $>4.4$ kcal/mol. Interestingly mutation of His$^{185}$ or Arg$^{265}$ each reduced the binding affinity by 2–3 kcal/mol, suggesting that if the contributions of these two residues are additive, the loss in binding energy by replacing the bridging nitrogen with S or O is equivalent to losing both interactions.

The x-ray structures of PfDHODH bound to the triazolopyrimidine-based inhibitors represent the first structural studies of the malarial enzyme bound to malaria-specific inhibitors, and as such they provide considerable new insight into species selective binding in this enzyme family. The inhibitor-binding pocket can be divided into site A (the H-bond pocket), which forms H-bonding interactions with the bound inhibitors, and site B, which is entirely hydrophobic (Fig. 4C). Both malaria and human-specific inhibitors interact with site A. Species selectivity of inhibitor binding arises from differences in the orientation of site B relative to site A within the protein structure. These differences have their basis in the species-specific amino acid composition of the inhibitor-binding pockets. For the triazolopyrimidine analogs, specificity for PfDHODH arises from the projection of an aromatic side chain from the N-1 position into the P$n$aphthyl pocket, which is blocked in the human structure. Whereas for brequinar the presence of the biphenyl group on the equivalent position to C-2 of DSM1 allows good interaction with the $h$$_{hydrophobic}$ pocket, this orientation cannot be accommodated by either the site B-binding pocket on PfDHODH. The structural basis for differential binding of A77 1726 is similar. The fact that residues that form H-bonds with the triazolopyrimidine ring (P$^{{\text{His}}^{185}}$ and P$^{{\text{Arg}}^{265}}$) are conserved in the human enzyme suggests inhibitors of hDHODH could also be developed based on this core scaffold.

The x-ray structures of PfDHODH bound to the triazolopyrimidine inhibitors provide significant insight into the observed SAR for this series. In addition to explaining the loss of activity upon replacing the bridging nitrogen N-1 with S or O, several other aspects of the SAR (17, 18) can now be readily understood. First we have observed that when the aromatic group is a phenyl, para substitutions yield good binding potency, whereas ortho substitutions are completely inactive. The PfDHODH-DSM74 structure shows that the ortho carbon atoms are 4.1 Å from the carbonyl oxygen of His$^{185}$ on one side and 4.0 Å from the CD1 of Leu$^{172}$ on the other. Thus based on the current structures there is not space in the pocket to accommodate ortho substituents, nor apparently is this region of the binding pocket capable of the conformational flexibility that would be needed to accommodate this substitution. Second, the completely hydrophobic nature of the P$n$aphthyl pocket explains why aromatic rings containing heteroatoms show reduced potency. Third, the close interaction between His$^{185}$ and the bridging nitrogen N-1 explains why a secondary nitrogen is required at this position and why the addition of a third substituent on the nitrogen reduces potency. Finally, the potential for stacking interactions between Phe$^{227}$ and Phe$^{188}$, in addition to the hydrophobic nature of the pocket, explains why large aromatic groups are favored.

In summary, x-ray structure determination of PfDHODH in complex to three triazolopyrimidine-based inhibitors has provided insight into the structural basis for potent and species-selective binding of this series of inhibitors. It has advanced our understanding of the emerging SAR for this promising lead series. Ongoing efforts to improve the potency and in vivo properties of the triazolopyrimidine-lead series will be greatly aided by these studies. More broadly, the results have provided insight into the structural requirements for designing species-selective inhibitors of either malarial or human DHODH that can be applied to scaffolds beyond the triazolopyrimidine analogs. Finally, the advantages of attacking plastic active sites in lead optimization offer general lessons for prioritizing structure-based protein-ligand interactions for pharmaceutical applications.

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