Identification and Characterization of Small Molecule Inhibitors of Plasmodium falciparum Dihydroorotate Dehydrogenase

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Plasmodium falciparum causes the most deadly form of malaria and accounts for over one million deaths annually. The malaria parasite is unable to salvage pyrimidines and relies on de novo biosynthesis for survival. Dihydroorotate dehydrogenase (DHOD), a mitochondrially localized flavoenzyme, catalyzes the rate-limiting step of this pathway and is therefore an attractive antimalarial chemotherapeutic target. Using a target-based high throughput screen, we have identified a series of potent, species-specific inhibitors of P. falciparum DHOD (pfDHOD) that are also efficacious against three cultured strains (3D7, HB3, and Dd2) of P. falciparum. The primary antimalarial mechanism of action of these compounds was confirmed to be inhibition of pfDHOD through a secondary assay with transgenic malaria parasites, and the structural basis for enzyme inhibition was explored through in silico structure-based docking and site-directed mutagenesis. Compound-mediated cytotoxicity was not observed with human dermal fibroblasts or renal epithelial cells. These data validate pfDHOD as an antimalarial drug target and provide chemical scaffolds with which to begin medicinal chemistry efforts.

The current suite of approved antimalarial drugs is limited to only a few targets within the malignant human malaria parasite, Plasmodium falciparum, a sophisticated eukaryotic organism with ~5,300 genes. Widespread resistance to mainstay therapies such as chloroquine (CQ)² (1–4), atovaquone (ATV) (5), pyrimethamine (Pyr) (6–8), and sulfadoxine (9–11) has created a need for new antimalarial agents. Here we address this issue through the identification and characterization of dihydroorotate dehydrogenase (DHOD) inhibitors with high specificity for the Plasmodium enzyme, submicromolar efficacy against cultured parasite strains, and no detectable cytotoxicity to human cells.

DHOD is a flavoenzyme that catalyzes the oxidation of L-dihydroorotate (L-DHO) to orotate as part of the fourth and rate-limiting step of the de novo pyrimidine biosynthetic pathway (Scheme 1) (12–15). The DHOD enzyme family can be separated by sequence homology into two broad classes that correlate with cellular localization and preference for electron acceptors (16–18). Both classes of enzyme perform a two-step reaction that most likely proceeds through a ping-pong mechanism (19–22). Gram-positive bacteria and the budding yeast Saccharomyces cerevisiae use a type 1 DHOD that is located in the cytosol and utilizes fumarate or NAD⁺ as an electron acceptor to reoxidize the flavin (FMN) prosthetic group in the second half-reaction of the redox process (13, 23–26). Type 2 DHODs are membrane-bound and generally found in eukaryotes and some Gram-negative bacteria (27–30). This class of enzyme uses quinones located in biological membranes as an electron acceptor (31). Specifically, eukaryotic type 2 DHODs are localized to the inner mitochondrial space and utilize the respiratory chain quinone coenzyme Qₙ (CoQₙ), where the number of isoprenoid units (n) is variable, as a final electron acceptor (32–35). Humans and all characterized members of the genus Plasmodium utilize a type 2 DHOD for de novo pyrimidine biosynthesis (34–38).

Pyrimidines are required for the biosynthesis of DNA, RNA, glycoproteins, and phospholipids. Most organisms possess both a salvage and de novo pyrimidine biosynthetic pathway; however, the P. falciparum genome lacks necessary components in the pyrimidine salvage pathway rendering the parasite entirely dependent on de novo biosynthesis (39, 40). Previous studies have shown that during the erythrocytic stages of P. falciparum development, the mitochondrial electron transport chain exists solely to regenerate CoQₙ to serve as an electron acceptor for DHOD (41). Although the salvage pathway for pyrimidines is generally able to fulfill the majority of metabolic

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§ The abbreviations used are: CQ, chloroquine; DHOD, dihydroorotate dehydrogenase; pfDHOD, P. falciparum DHOD; ATV, atovaquone; PyR, pyrimethamine; L-DHO, L-dihydroorotate; CoQₙ, decylubiquinone; DCIP, 2,6-dichlorophenol-indophenol; FeCy, ferricyanide; HTS, high throughput screen; hsDHOD, Homo sapiens DHOD; scDHOD, S. cerevisiae DHOD.
Inhibitors of pfDHOD

needs in human cells, rapidly dividing cells such as activated T- and B-lymphocytes require de novo biosynthesis for sustained growth. The active metabolite of the Food and Drug Administration-approved immunomodulatory drug leflunomide, A77 1726, is an inhibitor of human DHOD (hsDHOD) that exploits this specific vulnerability for the treatment of rheumatoid arthritis (42–45). A number of other DHOD inhibitors have been described for Helicobacter pylori and Escherichia coli that exhibit species selectivity with respect to the human enzyme (46, 47). Furthermore, species selectivity in designing small molecule inhibitors of P. falciparum DHOD (pfDHOD) should be possible as x-ray crystal structures of hsDHOD and pfDHOD in complex with A77 1726 and orotate show variability in both sequence and alignment of residues near the inhibitor-binding site (48, 49). As a consequence of this structural variability, A77 1726 is a potent inhibitor of the human enzyme but a very poor inhibitor of pfDHOD (50). Combined, these findings indicate that small molecule inhibitors of pfDHOD could be useful chemotherapeutic agents for malaria.

Although our primary goal was to identify inhibitors of pfDHOD that possessed activity against P. falciparum, we also tested inhibitors for activity against DHOD from the benign human malaria parasite Plasmodium vivax and the causative agent of rodent malaria, Plasmodium berghei. P. vivax malaria poses an enormous economic burden throughout many developing countries (51), and it would be advantageous to develop a single drug with efficacy against both pfDHOD and P. vivax DHOD (pvDHOD). The rodent malaria enzyme was examined because the accepted drug development pathway for P. falciparum malaria uses a P. berghei mouse model. All three DHOD proteins share significant homology, and thus it was hypothesized that candidate pfDHOD inhibitors may be efficacious against DHOD enzymes from other Plasmodium spp.

**EXPERIMENTAL PROCEDURES**

**DHOD Plasmid Construction—pfDHOD** was subcloned into the pET101D vector (Invitrogen) from a previously described codon-optimized, synthetic gene encoding amino acids 159–565 (49). Site-directed mutant pfDHOD-pET22b expression constructs (H185A, F188A, F227A, R265A, I272A, TYR-528A, and L531A) were kindly provided by M. Phillips from the University of Texas Southwestern Medical Center (50, 52, 53). Both wild-type and mutant pfDHOD constructs were in-frame with a C-terminal His6 tag. Full-length, codon-optimized DNA encoding the pdDHOD and pvDHOD genes were donated by GlaxoSmithKline (Philadelphia) and subcloned into the pET101D expression vector in-frame with the C-terminal His6 tag. To improve solubility, the P. berghei and P. vivax DHOD genes were truncated to include amino acids 132–518 and 160–573, respectively, based upon sequence alignment with pfDHOD. hsDHOD was subcloned into the pET101D expression vector in an analogous manner to the Plasmodium DHOD orthologs from a previously described expression plasmid with the final construct encoding amino acids 30–396 (48). Full-length S. cerevisiae DHOD (scDHOD) was amplified from genomic DNA and cloned directly into the pET101D expression vector in-frame with the C-terminal His6 tag. The DHOD open reading frames of all orthologs were sequenced in their entirety.

**DHOD Expression and Purification—E. coli BL-21(DE3) cells** (Invitrogen) transformed with either the wild-type or mutant pfDHOD, pvDHOD, pdDHOD, hsDHOD, or scDHOD expression constructs were grown in Terrific Broth with 100 μg/ml ampicillin at 30 °C. Protein expression was induced at A600 ~0.8 with the addition of isopropyl β-D-thiogalactopyranoside at a final concentration of 200 μM. The cultures were grown overnight at 22 °C. Cells were pelleted by centrifugation at 7,000 × g at 4 °C and frozen at −20 °C for later use. All subsequent purification steps were performed at 4 °C. Bacterial pellets were thawed in lysis buffer (50 mM HEPES (pH 7.5), 50 mM NaCl, 40 mM imidazole, 0.1% Triton X-100) supplemented with Complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science). The cells were lysed by sonication and clarified by centrifugation at 48,000 × g for 20 min. The supernatant was applied to a low pressure column of nickel-nitrilotriacetic acid resin (Invitrogen) pre-equilibrated in Buffer A (50 mM HEPES (pH 7.5), 50 mM NaCl, 40 mM imidazole, 0.1% Triton X-100, 5 mM 2-mercaptoethanol). The column was washed with Buffer A until the A280 reached a stable base line; bound DHOD was eluted with Buffer B (50 mM HEPES (pH 7.5), 500 mM NaCl, 400 mM imidazole, 0.1% Triton X-100, 5 mM 2-mercaptoethanol). The eluted protein was serially dia lyzed against Buffer C (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Purity was assessed by SDS-PAGE and found to be greater than 85% for all DHOD enzymes. Total protein concentration was determined by Bradford analysis. DHOD requires a bound FMN cofactor for catalysis, and thus the concentration of FMN should equal the concentration of active protein (36, 54). FMN concentration was measured spectrophotometrically at 445 nm (ε445 = 12.5 mM⁻¹ cm⁻¹) by heat-denaturing the protein to release bound cofactor. Purified DHOD was aliquoted, flash-frozen in liquid N2, and stored at −80 °C for later use.

**High Throughput Screen to Identify pfDHOD Inhibitors**—The chromogen reduction assay was adapted for a high throughput screen (HTS) for inhibitors of pfDHOD activity in 384-well plates based upon previous studies (53). The assay was performed in a final volume of 50 μl of 100 mM HEPES (pH 8.0), 150 mM NaCl, 5% glycerol, 0.05% Triton X-100, 175 μM DHO, 11 μM decylubiquinone (CoQ10), 95 μM 2,6-dichlorophenol-indophenol (DCIP), and 10 nM pfDHOD. Compounds from the Genzyme Corp. (Cambridge, MA) Small Molecule Library, comprised of 208,000 diverse, largely commercially available molecules, were pin-transferred into the 384-well assay plates to a final drug concentration of 10 μM for the initial screen. The reaction proceeded at room temperature for 20 min after which A600 was measured using an automated microplate reader. The quality of data from the final assay was assessed using a statistical measure described by Zhang et al. (55) where the Z-factor = 0.6; a Z-factor between 0.5 and 1 is generally favorable for an HTS. Primary hits were defined as compounds that inhibited ≥70% of pfDHOD activity at the initial screening concentration of 10 μM. These compounds were re-tested at a concentration of 1 μM, and those that inhibited ≥50% of enzymatic activ-
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Re-screening of Primary Hits from pfDHOD Screen and Chemical Analogs—Analogs of primary hits were purchased, as available, from ChemDiv (San Diego) and Maybridge (Cornwall, UK). The purity of these compounds was greater than 90%. The active metabolite of leflunomide, A77 1726, was purchased from Axxora Life Sciences (San Diego). IC50 values, with respect to pfDHOD activity for candidate compounds, were calculated using the aforementioned 384-well based chromogen reduction assay with 16–24 different drug concentrations and reagent concentrations of 200 μM L-DHO, 18 μM CoQD, 100 μM DCIP, and 10 nM pfDHOD. The concentration of CoQD is approximately equivalent to the reported Km value, and the concentration of L-DHO is in excess (49, 50). Select compounds were assayed in an analogous manner for the inhibition of site-directed pfDHOD mutants, pvDHOD, pbDHOD, and hsDHOD. The reported Km value of CoQD for pbDHOD, pvDHOD, and pfDHOD variants with point mutations at H185A, F188A, F227A, R265A, I272A, or Y528A, L531A was approximately equivalent to that of wild-type pfDHOD, and thus, the substrate concentrations used to assay enzyme activity and calculate IC50 values were kept constant between the various proteins that were examined (37, 52, 56, 57). Inhibition of scDHOD, a type 1 DHOD, was evaluated using the DCIP assay described above for type 2 DHOD enzymes except that 115 μM fumarate was used in lieu of CoQD. The concentration of fumarate corresponds with the reported substrate Km value, whereas the concentration of L-DHO is in excess (58).

DHOD activity using FeCy as an electron acceptor was measured continuously in a UV-transparent cuvette. The assay was performed in a final volume of 100 μl of 100 mM HEPES (pH 8.0), 150 mM NaCl, 5% glycerol, 0.05% Triton X-100, 200 μM L-DHO, 100 μM ferricyanide (FeCy). Both the reduction of FeCy and the formation of orotate were monitored at 420 nm (ε420 = 1 mM⁻¹ cm⁻¹) and 290 nm (ε290 = 2 mM⁻¹ cm⁻¹), respectively, noting that two molecules of FeCy are reduced for two molecules of L-DHO. As with the previous assay, the reaction was performed at 25 °C and initiated with the addition of 5–10 nm of enzyme.

Inhibition of in Vitro Cultured P. falciparum Parasites—P. falciparum strains D10, 3D7, HB3, and Dd2 were obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). The parasite strains were maintained in vitro by the method of Trager and Jensen (59). Cultures were maintained in fresh O-positive human erythrocytes (Interstate Blood Bank, Memphis, TN) in complete medium containing 50 ml of human O-positive serum (Interstate Blood Bank), 2.5 g of AlbuMAX II (Invitrogen), 0.5 ml of gentamicin (Sigma), 5.94 g of HEPES (Sigma), 2.01 g of sodium bicarbonate (Sigma), 0.050 g of hypoxanthine (Sigma), and 10.44 g of RPMI 1640 medium (JRH Biosciences, Lenexa, KS) per liter at pH 6.75. The parasite-infected erythrocytes were incubated at 37°C in a gas environment of 5% CO2, 1% O2, and 94% N2.

A transgenic P. falciparum strain expressing yeast DHOD was generated by amplification of full-length scDHOD from S. cerevisiae genomic DNA and subsequent cloning into an episomal expression vector derived from the pHHM plasmid (60). The resulting plasmid was transfected into the D10 strain of P. falciparum by standard methods and grown under selection with WR99210 (Jacobs Pharmaceutical Co., Princeton, NJ).

Inhibition of P. falciparum growth in the presence of drug was assessed by the relative reduction of [3H]hypoxanthine uptake (61, 62). Briefly, synchronized ring stage parasites at 1% parasitemia and 2% hematocrit were dispensed into 96-well plates containing dilutions of the candidate compounds. After 24 h, 0.4 μCi of [3H]hypoxanthine was added to each well, and the parasites were incubated for another 48 h at 37°C. P. falciparum parasites were harvested onto glass fiber filters, and the amount of [3H]hypoxanthine uptake was assessed by scintillation counting.

Structure-based Docking—All programs were from Suite 2008 from Schrödinger LLC (New York). A crystal structure of A77 1726 bound to pfDHOD (Protein Data Bank code 1TV5) was optimized for docking studies with Protein Preparation Wizard. Three-dimensional ligand structures were generated via LigPrep. Ligands were fit to the crystal structure with Glide version 50111 in extra precision (XP) mode. Images were obtained through the Maestro interface.

Cytotoxicity of Candidate Compounds—Human dermal fibroblasts were cultured in Earle’s minimal essential medium (Cambrex Inc., East Rutherford, NJ) supplemented with 15% fetal bovine serum (HyClone Laboratories, Logan, UT), 2× Complete Amino Acids (Sigma), 2× MEM vitamins (Sigma), and penicillin-streptomycin/β-glutamine (Cambrex). Cells were grown to 70–80% confluency, diluted to 0.25–0.3 × 10^5 cells/ml in fresh medium, and 200 μl aliquoted into 96-well flat bottom tissue culture treated plates. Candidate compounds were added after 24 h of incubation at 37°C and 5% CO2. Cell viability was assessed after 96 h by staining with Alamar Blue (BIOSOURCE). Cytotoxicity to renal proximal tubule epithelial cells was evaluated using the same method as described above except that the renal epithelial growth medium bullet kit (Clonetics, San Diego, CA) was used in lieu of Earle’s minimal essential medium and the renal epithelial cells were diluted to 1.5×10^4 cells/ml prior to aliquoting into 96-well plates.

RESULTS

High Throughput Screen to Identify Inhibitors of pfDHOD—DHOD catalyzes the oxidation of L-DHO to orotate via a ping-pong mechanism using a FMN cofactor that is reoxidized by the second substrate, CoQD (Scheme 1). Enzymatic activity of
DHOD can be assessed in the presence of L-DHO and CoQ₉ by directly measuring the formation of orotate or coupling the assay to the chromogen DCIP. The latter colorimetric assay was adapted for an HTS of recombinant pfDHOD in 384-well plates in a manner similar to that described by Baldwin et al. (53). CoQ₉ was chosen as the second substrate as it is significantly more soluble than ubiquinone (CoQ₁₀) and has been previously described to be efficiently utilized by pfDHOD (50, 63). The concentration of L-DHO used in the HTS was approximately equivalent to the reported $K_m$ value for both hsDHOD and pfDHOD, and the concentration of L-DHO was in slight excess (49, 50). The optimized screening assay afforded a Z-factor $\approx 0.6$, and a value between 0.5 and 1 is generally considered favorable for an HTS (55).

The Genzyme Corp. chemical library of 208,000 compounds was screened for inhibitors of pfDHOD at an initial drug concentration of 10 µM; 689 (0.3%) compounds were found to inhibit $\geq 70\%$ of pfDHOD activity when compared with control reactions containing no drug. These compounds were cherry-picked and re-tested at a concentration of 1 µM yielding 55 compounds that inhibited $\geq 50\%$ of pfDHOD activity. Dose-effect curves identified 38 (0.02%) compounds with submicromolar IC₅₀ values with respect to pfDHOD activity. The long, hydrophobic quinone binding tunnel of pfDHOD likely accommodates a variety of ligands, which is consistent with the core structural diversity of compounds identified that inhibit the enzyme (49). Although many different core structures were represented among the most potent inhibitors, a larger than expected number of amide moieties conjugated to large aromatic rings were classified as hits from the HTS. This same bias was also observed by Baldwin et al. (53) in a comparable HTS for pfDHOD inhibitors.

### Inhibition of Cultured P. falciparum Parasites by DHOD Inhibitors

From the 38 pfDHOD inhibitors that were evaluated for antimalarial efficacy using P. falciparum 3D7 as the test strain, five compounds were found to have submicromolar IC₅₀ values (Table 1). These compounds were then tested for inhibitory activity against multidrug-resistant HB3 and Dd2 P. falciparum strains. The 3D7 strain is sensitive to common antimalarials such as CQ and PYR and thus is ideal for preliminary evaluation of candidate drugs. The HB3 and Dd2 strains are derived from patient isolates and are resistant to CQ and/or PYR (HB3, CQ-sensitive, PYR-resistant; Dd2, CQ-resistant, PYR-resistant) (64, 65). Antimalarial efficacy of the pfDHOD inhibitors was consistent between the three different strains of P. falciparum. Compound-mediated cytotoxicity toward human dermal fibroblasts or renal epithelial cells was not observed at concentrations up to 60 µM.

### Inhibitory Activity and Species Selectivity of Screening Hits against DHOD Orthologs

All five pfDHOD inhibitors were found to possess antimalarial activity greater than 20-fold selective for pfDHOD over hsDHOD (Table 1). Compounds 2-5 were also equally efficacious against pbDHOD and pvDHOD, enzymes that are highly homologous to pfDHOD (72 and 65% identity, respectively).

| Compound | PfDHOD IC₅₀ (µM) | PbDHOD IC₅₀ (µM) | PvDHOD IC₅₀ (µM) | HsDHOD IC₅₀ (µM) | 3D7 Proliferation IC₅₀ (µM) | Dd2 Proliferation IC₅₀ (µM) | HB3 Proliferation IC₅₀ (µM) |
|----------|------------------|------------------|------------------|------------------|----------------------------|-----------------------------|-----------------------------|
| 1        | 0.93 ± 0.1       | 0.56 ± 0.07      | 5.6 ± 0.4        | > 30             | 0.32 ± 0.03                | 0.25 ± 0.02                 | 0.25 ± 0.01                 |
| 2        | 0.34 ± 0.1       | 0.11 ± 0.01      | 0.51 ± 0.03      | > 30             | 0.29 ± 0.02                | 0.29 ± 0.02                 | 0.24 ± 0.01                 |
| 3        | 0.042 ± 0.007    | 0.06 ± 0.007     | 0.42 ± 0.02      | > 30             | 0.49 ± 0.03                | 0.48 ± 0.02                 | 0.32 ± 0.02                 |
| 4        | 0.03 ± 0.06      | 0.38 ± 0.06      | 0.28 ± 0.01      | 7.9 ± 0.4        | 0.89 ± 0.07                | 0.89 ± 0.09                 | 0.62 ± 0.03                 |
| 5        | 0.083 ± 0.01     | 0.06 ± 0.007     | 0.04 ± 0.002     | > 30             | 0.77 ± 0.08                | 0.69 ± 0.03                 | 0.49 ± 0.04                 |

1 Errors represent the standard error of the fit for at least three determinations using 16–24 different drug concentrations.
2 IC₅₀ values were determined using a continuous assay where the reagent concentrations were 200 µM L-DHO, 18 µM CoQ₉, 100 µM DCIP, and 10 nM DHOD.
3 Antimalarial efficacy was calculated from dose-effect curves based on the relative reduction of [³H]hypoxanthine uptake by infected cultures.
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**TABLE 2**
Comparison of small molecule inhibition of wild-type versus mutant pfDHOD

| Compound | Fold change in IC\textsubscript{50} with respect to wild-type pfDHOD* | R = | IC\textsubscript{50} Values \( \mu M \) |
|----------|-------------------------------------------------|-----|-----------------|
|          | Wild type                                      |     | pfDHOD Activity | P. falciparum 3D7 Proliferation | CoQ\textsubscript{n} P. falciparum 3D7 Proliferation |
| 1        | H185A                                          | 1   | >100            | 0.42 ± 0.007                  | 0.71 ± 0.05 |
| 2        | F188A                                          | 2   | 0.6             | 0.46 ± 0.05                  | 0.11 ± 0.1 |
| 3        | F227A                                          | 16  | 1.1             | >35                          | >10         |
| 4        | R265A                                          | 5.4 | 0.8             | >35                          | >10         |
| 5        | I272A                                          | 5.9 | 1.2             | >55                          | >10         |
| A77 1726 | Y528A                                          | 5.9 | 0.8             | >15                          | >10         |

*IC\textsubscript{50} values were determined using a continuous assay where the reagent concentrations were 200 \mu M 1-DHO, 18 \mu M CoQ\textsubscript{n}, 100 \mu M DCIP, and 10 nM pfDHOD. Data obtained using mutant pfDHOD proteins were normalized to wild-type enzyme and represent at least three determinations using 16 different drug concentrations.

**TABLE 3**
Structure-activity relationship of compound 3

| Compound | IC\textsubscript{50} Values \( \mu M \) |
|----------|-----------------|
| 3        | 0.42 ± 0.007    |
| 6        | 0.46 ± 0.05     |
| 7        | >30             |
| 8        | >30             |
| 9        | >30             |
| 10       | 0.7 ± 0.09      |
| 11       | 0.17 ± 0.03     |

*Enzyme inhibition was evaluated using the DCIP assay where the reagent concentrations were 200 \mu M 1-DHO, 18 \mu M CoQ\textsubscript{n}, 100 \mu M DCIP, and 10 nM pfDHOD. Parasite proliferation was quantified by the relative reduction of \[^{3}H\]hypoxanthine uptake by P. falciparum-infected erythrocytes. Errors represent the standard error of the fit for at least three determinations using 16 different drug concentrations.

Mode of pfDHOD Inhibition—Calculation of meaningful inhibition constants by steady-state kinetic analysis is difficult when examining two-substrate enzymatic reactions and is further complicated when studying a ping-pong mechanism such as that catalyzed by DHOD. Malinquist et al. (52) demonstrated that the \( K_{\text{cat(app)}} \) for CoQ\textsubscript{n} to pfDHOD likely reflects multiple kinetic steps and thus does not necessarily represent a traditional \( K_m \) value. In the absence of CoQ\textsubscript{n}, the nonspecific inorganic oxidants FeCy or O\textsubscript{2} can reoxidize the FMN cofactor presumably via a direct electron-transfer pathway (52). Therefore, we evaluated compounds 1-5 for inhibition of pfDHOD activity using FeCy as the terminal electron acceptor. None of the compounds significantly inhibited this reaction at a concentration of 50 \mu M, whereas pfDHOD activity was fully inhibited using the endogenous substrate with an equal inhibitor concentration (data not shown). Inhibition of hsDHOD activity by A77 1726 with FeCy as the final electron acceptor was minimal in contrast to complete inhibition of catalysis afforded when using CoQ\textsubscript{n} as the second substrate. Ineffective inhibition of DHOD activity when using FeCy as an artificial substrate suggests a mechanism of inhibition that is specific to the transfer of electrons from FMN to CoQ\textsubscript{n}, reminiscent of an inhibitor situated in the long, hydrophobic tunnel.

Mapping of the Protein-Ligand-binding Site—Although A77 1726 is a fairly weak inhibitor of pfDHOD activity, the protein-ligand binding pocket has been structurally defined by x-ray crystallography (48, 49, 66, 117). Based upon this structural analysis and reports of other DHOD orthologs, it was hypothesized that the A77 1726-binding site may lie in the same binding pocket as A77 1726. The CoQ\textsubscript{n} binding site was previously thought to overlap with that of A77 1726 (48, 49, 66); however, Malinquist et al. (52) have shown that the two binding sites are different. CoQ\textsubscript{n} and 2 share significant structural homology and thus compound 2 may occupy the still undefined but distinct CoQ\textsubscript{n}-binding site.

In Silico Docking of pfDHOD Ligands—As a test case, a series of indole derivatives of compound 3 were purchased from commercial vendors. These were tested for inhibitory activity against purified pfDHOD protein and antimalarial efficacy (Table 3). Analysis of these compounds found that the bicyclic structure was necessary for activity; indoles and benzimidazoles performed well, whereas piperidine and morpholine rings fared poorly. Docking simulations provided a structural basis for these findings. The multiple ring substituents were better able to fill a hydrophobic pocket in the proposed binding site (Fig. 1) and, in particular, compound 3 formed hydrogen bonds to critical pfDHOD residues (supplemental Fig. 1).

Antimalarial Mechanism of Action—Although compounds 1-5 are submicromolar inhibitors of pfDHOD enzymatic activity, their inhibitory action against the whole parasite could, in principle, involve pathways other than pfDHOD. A transgenic P. falciparum strain expressing a cytoplasmic type 1 DHOD from S. cerevisiae, in addition to its endogenous mitochondrial pfDHOD, was generated to rule out this possibility. Painter et al. (41) had previously shown in an analogous experiment that the role of the mitochondrial electron potential, maintained in part by the cytochrome bc\textsubscript{2} complex, in the asexual stage of P. falciparum growth was mainly to reoxidize CoQ\textsubscript{n}, to sustain pfDHOD activity and subsequent de novo pyrimidine biosynthesis (41, 69). Consistent with this observation, the dose-effect curve for the D10-scDHOD parasite strain grown in the pres-
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Although the dose-effect profile of compound 1 with regard to the scDHOD-expressing parasites was shifted in comparison with the parent strain, significant inhibition of the transgenic parasite proliferation ensued at drug concentrations above 5 \( \mu \text{M} \). The antimalarial activity of compound 1 may therefore involve multiple targets in addition to pfDHOD.

DISCUSSION

The continued development of novel antimalarial chemotherapies, particularly those aimed at new pathways, is necessary for the successful treatment of malaria as resistance to presently utilized drugs becomes more widespread. To address this need, we have focused our drug discovery efforts on pfDHOD, the rate-limiting enzyme in the de novo pyrimidine biosynthetic pathway. Unlike its human host, the malaria parasite does not possess a salvage pathway for pyrimidines and must rely on de novo biosynthesis for its metabolic needs. pfDHOD is therefore likely to be an essential enzyme, and homologs from other species have previously been shown to be amenable to small molecule inhibition.

A library of 208,000 compounds was screened for enzyme inhibitors of pfDHOD. A total of 38 compounds were identified as submicromolar inhibitors of which 33 were specific for pfDHOD as compared with hsDHOD. The low observed hit rate of 0.018\% is a consequence of the stringent screening criteria. Baldwin et al. (53) performed an analogous HTS in which a relatively high proportion of hits contained amide and urea moieties conjugated to large aromatics. We too observed a similar selection of amide moieties conjugated to large aromatic rings, but we did not identify any of the same molecules indicating that the two different chemical libraries likely have few compounds and scaffolds in common.

Potent, species-selective inhibitors of pfDHOD have been described previously, but in vitro antimalarial efficacy has thus far been elusive. Here we identified five inhibitors of pfDHOD that also possess submicromolar antimalarial activity. To the best of our knowledge, this is first study of an HTS for pfDHOD.

No inhibition of scDHOD enzymatic activity was observed in the presence of compounds 1–5 at 30 \( \mu \text{M} \). Dose-effect curves measuring parasite proliferation for both the parent and D10-scDHOD strains grown in the presence of compounds 1–5 are shown in supplemental Fig. 2. The antimalarial efficacy of compounds 2–5 against the D10-scDHOD strain is significantly reduced when compared with the parent parasite strain. Specifically, growth inhibition of the transgenic parasite strain by these four compounds affords a biphasic drug-effect profile where the IC\(_{50}\) values for the second phase of curve are greater than 10 \( \mu \text{M} \) (Table 4). This rescued death phenotype concomitant with the addition of exogenous scDHOD argues that pfDHOD is the likely and primary in vivo target of compounds 2–5.

TABLE 4

| Compound | IC\(_{50}\) of P. falciparum proliferation (\( \mu \text{M} \)) |
|----------|--------------------------------------------------|
|          | D10 D10-scDHOD*                                |
| 1        | 0.30 ± 0.05 8.8 ± 1.2                         |
| 2        | 0.37 ± 0.1 >10                                 |
| 3        | 0.61 ± 0.03 >10                               |
| 4        | 0.95 ± 0.07 >10                               |
| 5        | 1.2 ± 0.06 >10                                |
| CQ       | 0.016 ± 0.001 0.013 ± 0.001                     |
| ATV      | 0.00042 ± 0.00005 >2                          |

*The IC\(_{50}\) values reported represent the second phase of the dose-effect curves shown in supplemental Fig. 2.
Inhibitors of pfDHOD

Inhibition profiles of wild-type versus mutant pfDHOD was used to localize the binding site of compounds 1, 3, 4, and 5 to be on or about that of A77 1726. Compound 2, however, is believed to bind a distinct site, likely that of the CoQ substrate based upon structural similarities.

Our findings confirm the value of pfDHOD as a target for antimalarial chemotherapy. The advancement of any of the candidate compounds presented here along a drug development path will require a significant investment in medicinal chemistry. Nevertheless, these chemical scaffolds provide a starting point for further development. Indeed, in silico modeling and structure-activity exploration of compound 3 and a series of structural analogs have afforded insight with regard to hit optimization.

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