High-Throughput Screening for Potent and Selective Inhibitors of
*Plasmodium falciparum* Dihydroorotate Dehydrogenase †

Jeffrey Baldwin§, Carolyn H. Michnoff¶, Nicholas A. Malmquist§, John White#, Michael G. Roth¶, Pradipsinh K. Rathod# and Margaret A. Phillips§*

§Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, Texas 75390-9041
¶Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas
#Department of Chemistry and Pathobiology, University of Washington, Seattle, WA 98195

*Author to whom all correspondence should be addressed. Tel: (214) 648-3637. Fax: (214) 648-9961. e-mail: margaret.phillips@UTSouthwestern.edu.

ABSTRACT

*Plasmodium falciparum* is the causative agent of the most serious and fatal malarial infections, and it has developed resistance to commonly employed chemotherapeutics. The *de novo* pyrimidine biosynthesis enzymes offer potential as targets for drug design because, unlike the host, the parasite does not have pyrimidine salvage pathways. Dihydroorotate dehydrogenase (DHODH) is a flavin-dependent mitochondrial enzyme that catalyzes the fourth reaction in this essential pathway; coenzyme Q (CoQ) is utilized as the oxidant. Potent and species-selective inhibitors of malarial DHODH were identified by high-throughput screening of a chemical library, which contained 220,000 drug-like molecules. These novel inhibitors represent a diverse range of chemical scaffolds, including a series of halogenated phenyl benzamide/naphthamides and urea-based compounds containing naphthyl or quinolinyl substituents. Inhibitors in these classes with IC$_{50}$ values below 600 nM were purified by HPLC, characterized by mass spectroscopy, and subjected to kinetic analysis against the parasite and human enzymes. The most active compound is a competitive inhibitor of CoQ with an IC$_{50}$ against malarial DHODH of 16 nM, and it is 12,500-fold less active against the human enzyme. Site-directed mutagenesis of residues in the CoQ-binding site significantly reduced inhibitor potency. The structural basis for the species selective enzyme inhibition is explained by the variable amino acid sequence in this binding site, making DHODH a particularly strong candidate for the development of new anti-malarial compounds.

INTRODUCTION

Malaria remains the dominant public health issue in many developing regions of the world and it causes millions of deaths annually with particularly highly mortality among children (1). Recent epidemiological data indicate that the global population exposed to malaria will continue to expand if there are no significant advancements to prevent and treat the disease (2). *Plasmodium falciparum* is responsible for most of the serious or fatal malaria occurrences, and it has developed resistance to commonly employed anti-malarial chemotherapeutics. Consequently, new drugs that can control infection are badly needed (3). While the redesign and synthetic optimization of existing antimalarials extends their lifetime as useful drugs, there is also a need to identify novel chemical scaffolds that inhibit existing and previously unexploited biological targets.

The *P. falciparum* enzymes active in *de novo* pyrimidine biosynthesis (Scheme 1) represent unique targets because the organism cannot salvage preformed pyrimidine bases or nucleosides (4-6). Dihydroorotate dehydrogenase (DHODH), the fourth enzyme in the pyrimidine biosynthetic pathway, has a number of properties that make it a particularly strong candidate as a new drug target in the parasite. Firstly, inhibitors of human DHODH already have proven efficacy for the treatment of rheumatoid arthritis in humans, demonstrating that the target pathway can be effectively blocked in vivo (7,8). Leflunomide, a pro-drug that is metabolized to generate the active immunosuppressive agent, and DHODH inhibitor A77-1726, is approved for use in this application (9-11). Secondly, the X-ray co-crystal structure of the human enzyme with A77-1726 (12) provides strong evidence that species
selective inhibitors of the enzyme can be developed. Alignment of the malarial DHODH sequence onto this structure indicated that more than half of the residues that are within van der Waals distance of A77-1726 are variable (Figure 1). Thirdly, species-selective pyrazole-based inhibitors of *H. pylori*, and of *E. coli* DHODH have been reported that were identified by high-throughput screening of chemical libraries (13,14). Finally, we previously demonstrated that *P. falciparum* DHODH is poorly inhibited by the potent human DHODH inhibitors redoxal, dichloroallyl lawson (DCL), and A77-1726 analogs (15). Thus, these studies suggest it should be feasible to exploit active-site differences to identify inhibitors that exhibit a high-degree of selectivity toward malarial DHODH.

High-throughput methodology provides a robust means to identify novel chemical scaffolds that have inhibitory activity against a target molecule. In this paper, we report the use of high-throughput screening technology of a 220,000 small drug-like molecule library to identify a number of potent and selective inhibitors of the *P. falciparum* DHODH. Two classes of molecules, based on biphenyl amide and urea structural scaffolds, were characterized in detail.

**MATERIALS AND METHODS**

**High-throughput Screening (HTS).**

**Chemical Library.** The compound collection was provided by the HTS screening center in the Department of Biochemistry at UTSWMC headed by Dr. Michael Roth. The collection is largely from commercial sources as illustrated by the described hits (Table 1). It is composed of 220,000 diverse, heterocyclic chemicals ranging in molecular weight from 250 – 550 Daltons, which were selected for drug-like properties.

**Assay methodology.** The cloning and expression of recombinant *P. falciparum* and human DHODH was described previously (15). The standard colorimetric DHODH continuous assay that monitors 2,6-dichloroindophenol (DCIP) reduction was adapted to an end-point assay in 384-well plates for compatibility with the high-throughput screening format. The assay solution containing 100 mM HEPES, pH 8.0, 150 mM NaCl, 10% glycerol, 0.05% Triton X-100, 20 µM CoQ0, 200 µM L-DHO, and 120 µM DCIP was prepared and 48 µL was transferred to each well using a Biomek FX robotic liquid handling device (Beckman Instruments). Each compound was prepared as a 300 µM stock in DMSO and out of that, 0.5 µL was transferred to the assay mixture to achieve approximately 3 µM in each well. The assay was started by addition of 2 µL of a 25X stock of enzyme prepared in 100 mM HEPES, pH 8.0, 300 mM NaCl, and 15% glycerol to give a final enzyme concentration between 20 – 60 nM in the assay well. The assay was allowed to progress for 20 minutes at room temperature and then the reaction was stopped by addition of 5 µL of 10% sodium dodecyl sulfate. The absorbance of each well was measured at 600 nm using a microplate reader (Bio-tek Instruments) and the data were exported to an Excel (Microsoft) spreadsheet for analysis. A ‘hit’ was recorded when a well had a measured absorbance greater than four standard deviations from the mean absorbance value of the no-drug DMSO control. This parameter corresponded to approximately 60% inhibition of enzymatic activity.

**Re-screening Hit Compounds.** Three sets of master plates were prepared at 300 µM, 60 µM, and 12 µM that consisted only of the compounds identified in the initial screen as potential inhibitors. The inhibition of the parasite enzyme was confirmed and the selective nature of the compounds was demonstrated by repeating the enzyme assay separately, for both the *P. falciparum* and human DHODH enzymes. The three sets of plates gave final concentrations of 3 µM, 0.6 µM, and 0.12 µM, thereby allowing a preliminary IC50 value to be calculated for each inhibitor according to equation 1.

\[
\frac{v_i - v_o}{I} = \frac{1}{IC_{50}}
\]

(Equation 1)

**Continuous Kinetic Rate Assays.** For several potent compounds, the IC50 values identified in the screen were also confirmed with traditional continuous assays that detect reduction of DCIP or direct production of orotate as described previously (15). The repeat assays used the same reagent concentrations as employed in the HTS assay.

**Inhibitor Solubility Studies.** Inhibitors (0.1 – 2.5 µM) were incubated with 10 nM enzyme in assay buffer lacking L-DHO for 6 minutes at room temperature. The mixture was centrifuged for 5 minutes at 17,000 rpm, the
supernantant transferred to a cuvette, and the reaction was initiated by the addition of L-DHO.

**Compound Verification.** Approximately 50 µg of each selected compound was applied to a 250 x 4.6 mm Nucleosil 5 µ C18 100 Å HPLC column (Varian) equilibrated with 0.05% trifluoroacetic acid/10% acetonitrile. The absorbance of each fraction was monitored at 254 nm and samples were eluted by increasing concentrations of acetonitrile as follows: 10-20 % ACN (5 minutes), 20 – 70% ACN (45 minutes), and 70-100% ACN (5 minutes). The molecular weight of the eluted material was determined by electrospray ionization mass spectrometry and inhibition of the pfDHODH enzyme was tested using the dominant peak. Additional fractions were also collected and tested before and after the major absorbing peak to confirm enzyme inhibition was not a result of small amount of contaminating material.

**Site-directed Mutagenesis.** The R265A and H185A (corresponding to R136 and H56 of human DHODH; Figure 1) variants of pfDHODH were prepared by PCR mutagenesis of the wildtype expression plasmid using a Quik-Change site-directed mutagenesis kit (Stratagene). The R265A construct was generated using primers CCACGTATTTTTGCAAGACGTTGAATCTAG and CTAGATTCAACGTCTGCAAAAAATACGTGG. Primers GTGAAATATGTGCTGACCTTTTTATTACC and CCTAGAATATGAGACGTTGAATCTAG were used to prepare the H185A variant (boldfaced bases represent the altered codon). Expression and purification was performed as described for the wild-type enzyme (15).

**In vitro testing against cultured malarial parasites.** To study inhibition of cell proliferation, 3H-hypoxanthine uptake was measured in drug-treated *P. falciparum* infected erythrocytes as previously described (16,17).

**RESULTS**

**High-throughput screen of a compound library to identify pfDHODH inhibitors.** The oxidation of L-dihydroorotic acid to orotic acid by DHODH occurs in the mitochondria of the parasite and is facilitated by the redox active cofactor flavin mononucleotide (18-20). The FMNH2 generated from the first half reaction is then re-oxidized by the second substrate ubiquinone (coenzyme Q10) to complete the catalytic cycle (21) as shown in scheme 1. An endpoint colorimetric assay of the enzyme was developed that was amenable to high-throughput screening in 384-well plates. It relied on the final oxidation of CoQ by the colorimetric reagent dichloroindophenol (DCIP) (see Materials and Methods). A chemical library of 220,000 compounds was screened to identify novel inhibitors of the *P. falciparum* enzyme (pfDHODH). The screen of the full library was performed with each compound present at a final concentration of 3 µM in the assay mixture. This yielded 1249 compounds that inhibited the enzyme by 60%; a hit rate of 0.6%. The results of a representative plate carrying one such inhibitor is displayed in Figure 2A.

Successful events in the initial screen led to the consolidation of select wells from the original library stock to generate a new, second generation of plates for re-screening and verification. Re-screening of these selected compounds was performed at three compound concentrations to allow calculation of a preliminary IC50 value, and to test for selectivity by comparison to the human enzyme. Importantly, almost all of the 1249 compounds identified in the original screen proved to be selective inhibitors of pfDHODH. Compounds with calculated IC50 values of less than 0.6 µM from this second screen were selected for further characterization. Halogenated phenyl benzamide/naphthamide compounds were the predominant class of inhibitor found in this group (Table 1). In addition, several urea-based compounds containing naphthyl or quinolinyl substituents were also shown to have good inhibitory activity. A number of other inhibitors from unique structural classes were also identified in the screen, and these are currently under evaluation.

**Verification of inhibition properties by kinetic rate assays.** The inhibitors shown in Table 1 were acquired in larger quantity from the indicated chemical suppliers to confirm the identity and inhibition properties of the molecules and to perform additional characterization. The IC50 values were measured by continuous kinetic rate assays using DCIP for detection at six different inhibitor concentrations for both the parasite and human enzymes. In addition,
pfDHODH was assayed directly by monitoring orotate production at 296 nm; this avoids potential artifacts from the DCIP reduction assay. Direct detection of orotate was not feasible for the higher concentrations of inhibitor necessary to inhibit the human enzyme because the compounds absorbed significantly at 296 nm. The IC\textsubscript{50}'s ranged from 20 – 800 nM in these assays confirming that the screen had identified a number of compounds that inhibit PfDHODH at nanomolar concentrations. The compounds also demonstrated selective inhibition of the parasite enzyme; the IC\textsubscript{50}'s for the human enzyme were from 70 – 12,500 fold higher than for PfDHODH. The largest difference in selectivity (12,500-fold) was observed for N-(3,5-Dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound 6), which was also the most potent inhibitor of the parasite enzyme (Table 1 and Figure 2).

**Verification of compound purity and identity.** To confirm the structural identity of the inhibitors, compounds were separated by reverse-phase HPLC and their molecular mass was verified by electrospray ionization mass spectrometry. A representative HPLC analysis for N-(3,5-dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound 6) is presented (Figure 3). Inhibition of PfDHODH was tested using material from peaks 1-4. Only material from peak 3 inhibited the enzymatic activity (data not shown; IC\textsubscript{50} = 24 ± 2 nM) and the mass (324) was consistent with the supplier expectation. These data confirm that compound 6 and not a degradation product or contaminant is responsible for the observed inhibition of PfDHODH. Similar analysis with other compounds from Table 1 also confirmed the structure and integrity of the hits from the high-throughput screen.

**Evaluation of compound selectivity.** A number of compounds identified either by high-throughput screening, or by structure-based computational approaches inhibit a wide range of target receptors, and thus they have been named promiscuous inhibitors (22-25). Many of these compounds form submicrometer aggregates that generate a large surface to which the enzyme is reversibly sequestered (23). Typically, these compounds have inhibition constants in the micromolar range. In addition, aggregation can be reversed through the addition of detergent such as Triton X-100 (24). We restricted our hits to molecules with IC\textsubscript{50} values less than 0.6 µM and the assays were performed in the presence of detergent. Nonetheless, further experiments to exclude compound promiscuity were performed by assaying activity following centrifugation of a pre-incubated solution of enzyme and inhibitor. If precipitation or aggregation of the enzyme leads to the observed inhibition, centrifugation would be expected to decrease the enzyme activity relative to the non-centrifuged samples and artificially lower the measured IC\textsubscript{50} values. Compounds 6, 8, and 12 were assayed in this manner and the calculated IC\textsubscript{50} values are in close agreement with the values obtained from the standard assays.

**Identification of inhibitor-binding sites on PfDHODH.** The X-ray structures of the two potent inhibitors A77-1726 and Brequinar in complex with the human enzyme demonstrate that both molecules are positioned in the pocket adjacent to the flavin cofactor (Figure 1). To test the hypothesis that our newly identified inhibitors bind to this variable site, we utilized site-directed mutagenesis to evaluate the effects of mutation of two conserved residues (R265A and H185A) on the inhibitory properties of the compounds. Inhibitor potency is affected by both substitutions providing good evidence that these compounds bind in the A77-1726 pocket (Table 2). Mutation of H185 had the largest effect and increased the IC\textsubscript{50} 500 – 1000-fold for all thirteen compounds, suggesting that this residue is an important binding determinant for the entire class. The corresponding residue (H56) from the human DHODH enzyme was also shown to be an important determinant for high-affinity binding of the anti-proliferative agent Brequinar (26). Mutation of R265 only affected the inhibitory properties of half of the compounds, and these effects were more modest (3 – 35-fold), suggesting the inhibitors have some variation in their binding modes.

The mutations also had modest effects on the steady-state kinetic parameters; the K\textsubscript{m} for CoQ\textsubscript{D} was increased 2-4-fold, and the k\textsubscript{cat} was reduced by a similar amount (Table 2B). Similar to the effects observed on inhibitor binding, mutation of H185 was more detrimental to activity then mutation of R265, causing an overall 16-fold reduction in k\textsubscript{cat}/K\textsubscript{m}. These results are consistent with the hypothesis that CoQ binds in the same site as A77-1726, and these newly identified inhibitors of the malarial enzyme. In order to further demonstrate that CoQ and the inhibitor binding sites overlap, the mode of
inhibition for N-(3,5-Dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound 6) was examined more extensively by determining IC\textsubscript{50} values at increasing concentrations of the CoQ\textsubscript{D} substrate (Figure 4). The observed linear increase in the IC\textsubscript{50} values with respect to substrate concentration is indicative of competitive inhibition, and therefore this compound likely binds to the CoQ site.

**Effects of DHODH inhibitors on cultured**

*P. falciparum* parasites. The compounds listed in Table 1 were tested for their ability to inhibit the growth of cultured parasites. Compound 1 had modest activity in whole cell assays against *P. falciparum*: proliferation was inhibited by 2% at 5 \( \mu \)M and by 20% at 10 \( \mu \)M. No significant growth inhibition was observed for any of the other compounds listed in Table 1 at concentrations ranging from to 10 - 100 \( \mu \)M, depending on the solubility limits. Thus, in spite of high potency against purified PfDHODH these compounds do not effectively inhibit parasite growth.

**DISCUSSION**

Current clinically useful antimalarial drugs were mostly discovered from leads that are several decades old and suffer from resistance, as well as cross-resistance amongst members of the same class of compounds (27-29). This, plus the high incidence of downstream drop-offs in drug development projects, makes it important to develop fresh leads for new antimalarials.

Malaria parasites, unlike the host mammalian cells, are completely dependent on de novo pyrimidine biosynthesis. Here, a high-throughput screening technology involving about 220,000 molecules was employed to identify new, potent and selective inhibitors of malarial DHODH, an important enzyme in de novo pyrimidine biosynthesis. One compound inhibited PfDHODH with an IC\textsubscript{50} of 16 nM and demonstrated a 12,500-fold difference in specificity for host versus parasite enzyme (Compound 6, Table 1). There were over a dozen compounds that inhibited below 100 nm and showed at least 100-fold selectivity.

The most potent and selective PfDHODH inhibitors displayed some structural similarities amongst themselves. There was an uncommon representation of amide and urea moieties conjugated to large aromatics (Table 1). It is likely that, structurally and electronically, the carbonyl groups satisfy an important binding requirement for potency. The planarity of the aromatics, plus the rotational resistance of the amides, also appeared to be important for potent inhibition of malaria enzyme. Some, but not all, of these molecular features were previously found in the potent human DHODH inhibitor A77-1726. While this compound carries an aromatic amine substitution, its acyl group is a small propeneamide instead of larger benzamide/naphthamide derivatives discovered in the present screen. The importance of the acyl size for host-parasite specificity is highlighted by A77-1726 analogs in which the propenamide was replaced with a quinoline; the resulting larger compounds were poor inhibitors of the human DHODH enzyme (30). Analogues of A77-1726, which are potent inhibitors of the human enzyme, are very poor inhibitors of pfDHODH (15). In parallel studies with other microorganisms, A77-1726 does not inhibit Gram-negative bacteria *H. pylori* DHODH or *E. coli* DHODH. Novel pyrazole-based compounds which selectively inhibit *H. pylori* or *E. coli* DHODH have little effect on the human enzyme, or cross-activity between the bacterial enzymes (13 90,14).

Seven of the potent and selective molecules (compounds 4-10) in the present screen were 2-nitro-3-methyl-benzamide derivatives. This is probably due to a good representation of this family of molecules in the source libraries, the high preference of the parasite enzyme for the 2-nitro-3-methyl-benzamide unit, and significant tolerance for change at the distal halogenated aromatic amines. Since the naphthamide derivatives (compounds 1-3) are also potent, it is clear that the parasite enzyme offers multiple solutions for selective recognition of DHODH. Compounds 11-13 represent yet another class of inhibitors. In this set, urea moieties carried a naphthyl or quinolinyl amine on one end and a substituted phenyl amine on the other end. While this urea scaffold could offer selectivity toward the parasite enzyme (compound 11), the screen failed to find potency that matched the best members of the amide-based compounds.

The amide-based as well as the urea-based inhibitors of the malaria enzyme appear to interact with the same part of the enzyme as traditional inhibitors of DHODH from other species. Primary structure alignment of DHODH enzymes from different organisms revealed that both variable and
highly conserved residues comprise the A77-
1726 binding site, suggesting that the variable 
residues form the basis for the observed species 
selective inhibition of pfDHODH (Figure 1). Site-
directed mutagenesis of two conserved residues in 
this pocket was used to determine if the newly 
discovered inhibitors of the malarial enzyme also 
bind this site. The H185A mutation decreased 
the affinity of many compounds by over 1,000 
fold, providing strong support for the hypothesis 
that these newly discovered inhibitors also bind 
this site (Table 2A). While mutagenesis has not 
been performed on any of the variable residues in 
this pocket, it seems likely that the structural basis 
for the species-selective inhibition is due to the 
differences in the shape and hydrophobicity of the 
pocket resulting from the variable amino acid 
composition. The mutant analysis also revealed 
some differences in binding modes between 
inhibitors: the R265A mutation affected some 
compounds by up to 35-fold, while there was no 
effect on others. This site is also thought to bind 
CoQ, though direct structural evidence for the 
CoQ site is still lacking (12,14,31). In support of a 
common binding site for the malaria enzyme, the 
most potent benzamidine derivatives (compound 6) 
appeared to be a simple competitive inhibitor with 
respect to CoQ utilization.

Comparison of the human DHODH 
crystal structures with the malaria DHODH amino 
acid sequence had suggested that malaria DHODH 
offers unusual opportunities for species-specific 
inhibitor binding. The present study formally 
demonstrates that it is possible to identify potent 
inhibitors of \textit{P. falciparum} DHODH that do not 
inhibit the human enzyme. Since malaria parasites 
lack enzymes for salvage of preformed 
pyrimidines, such compounds have enormous 
potential for selectivity at the cellular level. 
However, the transition from enzyme inhibitors to 
clinically useful antimalarials will, of course, offer 
new challenges. A useful drug must be effective 
against whole cells, exhibit low toxicity toward 
host cells, and have a desirable pharmacokinetic 
profile. While the compounds shown in table 1 
are excellent inhibitors of PfDHODH activity, 
they had weak antimalarial activity in cell-based 
assays. In principle, one possible explanation for 
the low \textit{in vivo} efficacy is the inefficient uptake of 
the compounds by the parasite. However, both 
amide and urea-derived molecules are known to 
function effectively as antiparasitic agents.

Niclosamide or Niclocide (5-Chloro-N-(2-chloro-
4-nitrophenyl)-2-hydroxybenzamide) is an 
antihelminthic compound used to treat worm 
infections. Its mode of action is to inhibit 
oxidative phosphorylation in the mitochondria of 
the parasite (32). This is encouraging since 
DHODH also has a mitochondrial location. In 
addition, diphenyl ureas that inhibit plasmepsins 
from \textit{P. falciparum} and \textit{P. vivax}, show selective 
killing of \textit{Plasmodium} versus mammalian cell 
lines (33). On this basis, a second generation 
library of the most potent inhibitors is likely to 
lead to compounds that are effective in whole 
cells. To this end, the synthetic simplicity of 
preparing biphenyl amide-derivatives should be 
helpful.
REFERENCES


FOOTNOTES.
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SCHEMES

Scheme 1. Reaction catalyzed by the de novo pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase (DHODH). The FMN cofactor facilitates conversion of L-dihydroorotate to the oxidized product orotate. The second substrate CoQ is then reduced by FMNH2 thereby regenerating oxidized flavin competent for further catalytic cycles.

FIGURE LEGENDS

Figure 1. Inhibitor binding-site of hDHODH. Amino acid residues within van der Waals’ distance of A77-1726 (shown in orange) are displayed. Non-conserved residues are colored purple whereas the remaining conserved residues are grey. The redox active cofactor FMN and orotate are shown in orange. The amino acid numbering and substitutions observed in the P. falciparum sequence are given in parenthesis.

Figure 2. A. Representative assay result from the HTS screen. Data illustrates the results from one 384 well plate. The endpoint DCIP assay was used in the screen. The starting Abs600 = 1.76 and the average Abs600 at the end of the incubation time in the absence of inhibitors was 1.14 ± 0.02. Hits were defined as values >4 standard deviations above the mean. On this plate one hit was identified with an Abs600 = 1.58, representing 70% inhibition of the reaction. B. Selective Inhibition of pfDHODH. A continuous assay was used to measure enzyme inhibition of N-(3,5-Dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound 6). The IC50 values were calculated as 0.016 ± 0.002 µM and 200 ± 14 µM for the P. falciparum (circles) and human (squares) DHODH enzymes, respectively.

Figure 3A. Analysis of acquired inhibitors. Compounds purchased from commercial sources were chromatographed by RP-HPLC equipped with a UV-absorbance detector (λ = 254 nm) to verify the active components. A representative chromatogram of N-(3,5-Dichloro-phenyl)-2-methyl-3-nitro-benzamide (compound 6) is shown. Material from peaks labeled 1-4 was collected and tested for inhibition of pfDHODH activity and only the sample originating from peak 3 inhibited the enzyme.

Figure 3B. Mass spectroscopy. Material from the active fraction (peak 3) was analyzed by electrospray ionization mass spectroscopy and confirms the predicted mass (324) of compound 6.
Figure 4. Mode of Inhibition. Steady state kinetic assays of compound 6 was employed to determine the mode of inhibition. The IC$_{50}$ values were determined at 5, 10, 50, 100, and 150 µM of substrate CoQ$_D$ using the direct assay that monitors orotate production. The linear increase is indicative of a competitive inhibitor with respect to CoQ$_D$. 
Assay\(^1\): IC\(_{50}\) values were determined using a continuous assay that monitors DCIP reduction at 600 nm. The reagent concentrations were 20 \(\mu\)M CoQ\(_0\), 200 \(\mu\)M L-DHO, 120 \(\mu\)M DCIP, and 10 nM enzyme.

Assay\(^2\): IC\(_{50}\) values were determined using a continuous assay that detects the oxidized product orotate at 296 nm. The reagent concentrations were 20 \(\mu\)M CoQ\(_0\), 200 \(\mu\)M L-DHO, and 10 nM enzyme.

Compound identity\(^3\): The original hit from the library was the 3-methyl-4-nitro derivative. This compound was not commercially available so the listed analog was used for the follow up analysis.

<table>
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<th>No.</th>
<th>Supplier</th>
<th>IC(_{50}), (\mu)M (HTS)</th>
<th>Assay(^1) IC(_{50}), (\mu)M</th>
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Table 1

\(^1\) Assay: IC\(_{50}\) values were determined using a continuous assay that monitors DCIP reduction at 600 nm. The reagent concentrations were 20 \(\mu\)M CoQ\(_0\), 200 \(\mu\)M L-DHO, 120 \(\mu\)M DCIP, and 10 nM enzyme.

\(^2\) Assay: IC\(_{50}\) values were determined using a continuous assay that detects the oxidized product orotate at 296 nm. The reagent concentrations were 20 \(\mu\)M CoQ\(_0\), 200 \(\mu\)M L-DHO, and 10 nM enzyme.

\(^3\) Compound identity: The original hit from the library was the 3-methyl-4-nitro derivative. This compound was not commercially available so the listed analog was used for the follow up analysis.
Table 2

A. 

<table>
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<tr>
<th>Compound</th>
<th>wtP/DHODH</th>
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<td>0.05 ± 0.01</td>
<td>0.08 ± 0.03 (2)</td>
<td>&gt;50 (&gt;1000)</td>
</tr>
<tr>
<td>2</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01 (1)</td>
<td>570 ± 130 (11,400)</td>
</tr>
<tr>
<td>3</td>
<td>0.47 ± 0.05</td>
<td>0.30 ± 0.08 (0.6)</td>
<td>210 ± 40 (440)</td>
</tr>
<tr>
<td>4</td>
<td>0.06 ± 0.01</td>
<td>1.2 ± 0.3 (2.0)</td>
<td>140 ± 30 (2400)</td>
</tr>
<tr>
<td>5</td>
<td>0.10 ± 0.02</td>
<td>0.50 ± 0.07 (5)</td>
<td>130 ± 30 (1300)</td>
</tr>
<tr>
<td>6</td>
<td>0.02 ± 0.002</td>
<td>0.40 ± 0.10 (20)</td>
<td>130 ± 20 (6300)</td>
</tr>
<tr>
<td>7</td>
<td>0.26 ± 0.02</td>
<td>0.82 ± 0.06 (3)</td>
<td>250 ± 60 (960)</td>
</tr>
<tr>
<td>8</td>
<td>0.08 ± 0.02</td>
<td>0.48 ± 0.09 (6)</td>
<td>180 ± 40 (2300)</td>
</tr>
<tr>
<td>9</td>
<td>0.08 ± 0.01</td>
<td>2.8 ± 0.5 (35)</td>
<td>60 ± 20 (790)</td>
</tr>
<tr>
<td>10</td>
<td>0.08 ± 0.01</td>
<td>0.37 ± 0.06 (5)</td>
<td>120 ± 20 (1600)</td>
</tr>
<tr>
<td>11</td>
<td>0.40 ± 0.04</td>
<td>0.34 ± 0.01 (1)</td>
<td>&gt;200 (&gt;500)</td>
</tr>
<tr>
<td>12</td>
<td>0.23 ± 0.05</td>
<td>0.24 ± 0.07 (1)</td>
<td>140 ± 30 (600)</td>
</tr>
<tr>
<td>13</td>
<td>0.78 ± 0.14</td>
<td>2.0 ± 0.4 (3)</td>
<td>&gt;400 (&gt;500)</td>
</tr>
</tbody>
</table>

Numbers in parenthesis are fold change in IC50 compared to wild-type.

B. 

<table>
<thead>
<tr>
<th>p/DHODH</th>
<th>( k_{\text{cat}} ), s(^{-1})</th>
<th>( K_M(\text{CoQD}) ), μM</th>
<th>( K_M(\text{L-DHO}) ), μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>7.8 ± 0.2</td>
<td>16 ± 1</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>R265A</td>
<td>4.6 ± 0.1</td>
<td>32 ± 5</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>H185A</td>
<td>1.7 ± 0.1</td>
<td>66 ± 8</td>
<td>123 ± 20</td>
</tr>
</tbody>
</table>
Scheme 1

L-Gln → L-DHO → DHODH → Orotate → UMP

HCO₃⁻  FMN  FMNH₂

ATP  CoQH₂  CoQ
Figure 1
Figure 2A

Figure 2B

[Compound 6], µM
Figure 3

A

M-H

B

(M-H)⁻

(M+H)⁺
Figure 4
Additions and Corrections

Vol. 280 (2005) 2220–2228

Rab13 mediates the continuous endocytic recycling of occludin to the cell surface.

Shinya Morimoto, Noriyuki Nishimura, Tomoya Terai, Shinji Manabe, Yasuyo Yamamoto, Wakako Shinahara, Hide-nori Miyake, Seiki Tashiro, Mitsuo Shimada, and Takuya Sasaki

Page 2227, Fig. 10C: It has come to our attention that the data for TfR and occludin in Fig.10C were identical to those in Fig.5B. This is because we inadvertently chose the wrong data sheet and mistook the data obtained from BHK cells (Fig. 5B) for those from MTD-1A cells in Fig.10C. As a consequence, the data for eight columns (four solid white and four solid gray bars) in Fig.10C were incorrect, although the legend was correct. The data under the heading Endocytosis should be changed as follows: endocytosis of TfR-mock, from 16.32 ± 1.52 to 16.44 ± 1.95% (left white bar); endocytosis of TfR-Rab13 Q67L, from 15.50 ± 2.45 to 18.79 ± 2.81% (right white bar); endocytosis of occludin-mock, from 20.98 ± 1.26 to 15.30 ± 2.79% (left gray bar); and endocytosis of occludin-Rab13 Q67L, from 19.61 ± 1.63 to 14.32 ± 2.33% (right gray bar). The data under the heading Recycling should be changed as follows: recycling of TfR-mock, from 63.89 ± 5.80 to 65.20 ± 9.44% (left white bar); recycling of TfR-Rab13 Q67L, from 66.70 ± 3.74 to 68.30 ± 10.08% (right white bar); recycling of occludin-mock, from 69.77 ± 3.81 to 64.82 ± 8.71% (left gray bar); and recycling of occludin-Rab13 Q67L, from 34.81 ± 7.04 to 37.65 ± 7.44% (right gray bar). The correct figure is shown here. Although this correction neither changes the interpretation of the data nor alters the conclusion of the paper, we sincerely apologize for any inconvenience our error may have caused.

Vol. 280 (2005) 21847–21853

High-throughput screening for potent and selective inhibitors of Plasmodium falciparum dihydroorotate dehydrogenase.

Jeffrey Baldwin, Carolyn H. Michnoff, Nicholas A. Malmquist, John White, Michael G. Roth, Pradipsinh K. Ratkhod, and Margaret A. Phillips

Page 21851, Table III: The \( k_{\text{cat}} \) value for the H185A mutant was incorrectly printed. It should read 1.7 ± 0.1. The correct table is shown here.

<table>
<thead>
<tr>
<th>pfDHODH</th>
<th>( k_{\text{cat}} )</th>
<th>( K_m(\text{CoQD}) )</th>
<th>( K_m(\text{L-DHO}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.8 ± 0.2</td>
<td>16 ± 1</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>R285A</td>
<td>4.6 ± 0.1</td>
<td>32 ± 5</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>H185A</td>
<td>1.7 ± 0.1</td>
<td>66 ± 8</td>
<td>123 ± 20</td>
</tr>
</tbody>
</table>

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
High-throughput screening for potent and selective inhibitors of Plasmodium falciparum dihydroorotate dehydrogenase
Jeffrey Baldwin, Carolyn H. Michnoff, Nicholas A. Malmquist, John White, Michael G. Roth, Pradipsinh K. Rathod and Margaret A. Phillips

J. Biol. Chem. published online March 28, 2005

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