The X-ray structure of *Plasmodium falciparum* dihydroorotate dehydrogenase bound to a potent and selective *N*-phenylbenzamide inhibitor reveals novel binding-site interactions

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Plasmodium species are protozoan parasites that are the causative agent of malaria. Malaria is a devastating disease, and its treatment and control have been hampered by the propensity of the parasite to become drug-resistant. Dihydroorotate dehydrogenase (DHODH) has been identified as a promising new target for the development of antimalarial agents. Here, the X-ray structure of *P. falciparum* DHODH bound to a potent and selective *N*-phenylbenzamide-based inhibitor (DSM59) is described at 2.3 Å resolution. The structure elucidates novel binding-site interactions and shows how conformational flexibility of the enzyme leads to the ability to bind diverse chemical structures with high affinity. This information provides new insight into the design of high-affinity DHODH inhibitors for the treatment of malaria.

1. Introduction

Malaria is endemic in over 90 countries and kills 0.63 million people yearly, with the majority of fatalities occurring in sub-Saharan African children under the age of five and pregnant woman (White *et al.*, 2014; Miller *et al.*, 2013; World Health Organization, 2012). Malaria is caused by protozoan parasites of the *Plasmodium* species, with *P. falciparum* and *P. vivax* accounting for most of the disease burden. Drug-therapy and insect-control programs have been the most effective methods for both preventing and treating malaria. However, despite a large arsenal of antimalarial agents, the emergence of drug resistance to the most effective drugs has hindered control of the disease (White, 2008; White *et al.*, 2014; Burrows *et al.*, 2014; Anthony *et al.*, 2012). Current malaria treatment programs rely on artemisinin-based combination therapies (ACTs), and their effectiveness has been instrumental in the significant global reduction in malaria deaths in the past decade (Eastman & Fidock, 2009). Unfortunately, artemisinin resistance has emerged in South East Asia, leading to increased parasite-clearance times and the need for extended treatment periods (Dondorp & Ringwald, 2013; Arie *et al.*, 2014; Ashley *et al.*, 2014). There is a clear need for new drug discovery to identify novel agents that can be used to treat malaria in the face of the continuing challenge of emerging drug resistance. In the past decade a significant portfolio of new chemical entities have been identified with antimalarial activity, and a number of compounds are currently in clinical development (Burrows *et al.*, 2013, 2014; Anthony *et al.*, 2012).

Dihydroorotate dehydrogenase (DHODH), an essential enzyme in the *de novo* pyrimidine-biosynthetic pathway (Fig. 1a), has emerged as one of a handful of new drug targets identified in this global effort to develop new antimalarial agents (Stocks *et al.*, 2014; Phillips & Rathod, 2010; Munier-Lehmann *et al.*, 2013). While most cells, including human cells, retain the ability to both synthesize pyrimidines *de novo* and to salvage preformed nucleosides and bases, the malaria parasite does not have salvage enzymes. Therefore, the *de novo* pathway is essential for the ability of the parasite to synthesize first uridine monophosphate (UMP; Fig. 1a) and then the additional pyrimidine nucleotides required for DNA and RNA biosynthesis and for cell growth. DHODH is one of the few new targets to have been validated through a target-based approach to lead identification; in contrast, most other compounds in the antimalarial portfolio were identified by phenotypic screens on cultured parasites (Burrows *et al.*, 2013, 2014; Anthony *et al.*, 2012). Our group used an enzyme-based high-throughput screening approach to identify novel inhibitors of *P. falciparum* DHODH (*Pf*DHODH; Baldwin *et al.*, 2005; Phillips *et al.*, 2008). We undertook a lead-optimization program around a class of triazolopyrimidine-based compounds and identified potent and species-selective compounds with excellent *in vivo* activity (*e.g.* DSM265 and DSM267; Fig. 1b; Coteron *et al.*, 2011; Deng *et al.*, 2009, 2014; Gujjar *et al.*, 2011;

Marwaha *et al.*, 2012). A compound (DSM265) from this series is currently in clinical development (<http://www.mmv.org>).

*Pf*DHODH is a mitochondrial enzyme that requires both flavin mononucleotide (FMN) and coenzyme Q (CoQ) to catalyze the reaction chemistry (Fig. 1a). Catalysis occurs *via* two half-reactions: the oxidation of dihydroorotate, catalyzed by the reduction of FMN, followed by the reoxidation of FMN by CoQ. In *Pf*DHODH, amino-acid residues 162–565 form the catalytic domain (β/α -barrel fold) and residues to the N-terminus of this domain are thought to be involved in anchoring the protein to the inner mitochondrial membrane, with the catalytic domain localized in the inner membrane space (Phillips & Rathod, 2010). Most described DHODH inhibitors bind to the presumed CoQ binding site, which sits adjacent to FMN between the β/α -barrel domain and the N-terminal α -helical membrane domain. Significant amino-acid sequence differences between the *Plasmodium* and human enzymes have been identified in this inhibitor-binding site that are thought to underpin the species-selectivity of these inhibitors, including for the triazolopyrimidine-based inhibitors of *Pf*DHODH (Deng *et al.*, 2009, 2014).

X-ray structural data for *Pf*DHODH in complex with various triazolopyrimidine analogs were essential to guide the medicinal chemistry effort leading to the identification of potent *Pf*DHODH inhibitors that retained excellent

Figure 1
(a) DHODH reaction scheme. (b) Chemical structures of *Pf*DHODH inhibitors.

Table 1

Comparison of binding affinity for *Pf*DHODH inhibitors.

Inhibitor	IC ₅₀ [†] (nM)	
	<i>Pf</i> DHODH	Human DHODH
DSM59	16	>100 000
DSM267	38	>100 000
Genz 667348	22	>30 000

[†] Data were taken from Baldwin *et al.* (2005), Booker *et al.* (2010) and Coteron *et al.* (2011).

selectivity *versus* the human enzyme (Coteron *et al.*, 2011; Deng *et al.*, 2009). These data, as well as the crystal structures of *Pf*DHODH bound to Genz 667348 (Fig. 1*b*), a *Pf*DHODH inhibitor from the thiophene carboxamide family (Booker *et al.*, 2010), and A77 1726, a clinically used human DHODH inhibitor (Hurt *et al.*, 2006), illustrated that the *Pf*DHODH inhibitor-binding site exhibits significant structural flexibility, contributing to its ability to bind multiple diverse chemical scaffolds with high affinity and selectivity. As a result of this flexibility, the inhibitors from these different structural classes bind *Pf*DHODH in overlapping but distinct binding modes.

In addition to the triazolopyrimidine series, we identified a series of *N*-phenylbenzamides that were selective *Pf*DHODH inhibitors, and one compound from this series (DSM59; Fig. 1*b* and Table 1) had the distinction of being the most potent of the identified screening hits against *Pf*DHODH (Baldwin *et al.*, 2005). Like the triazolopyrimidines, DSM59 is also a highly selective *Pf*DHODH inhibitor that does not inhibit the human enzyme. The *N*-phenylbenzamides lack activity against *P. falciparum* parasites, likely owing to poor cell permeability,

Table 2

X-ray diffraction data and refinement statistics for *Pf*DHODH–DSM59.

Values in parentheses are for the highest resolution shell.

Data collection	
Resolution (Å)	50.0–2.3
Space group	<i>P</i> 6 ₄
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 85.0, <i>c</i> = 137.7, α = β = 90, γ = 120
Unique reflections	25061
Completeness (%)	99.9 (100)
Multiplicity	8.2 (8.2)
<i>I</i> / <i>σ</i> (<i>I</i>)	24.5 (2.0)
<i>R</i> _{merge} (%)	8.1 (0.94)
CC _{1/2} [†]	(0.618)
Refinement	
<i>R</i> factor/ <i>R</i> _{free} (%)	18.8/24.7
No. of atoms	3093
No. of water atoms	50
<i>B</i> value (Å ²)	
Protein	39.4
Water	58.1
FMN	52.1
DSM59	65.1
R.m.s. deviations	
Bond lengths (Å)	0.017
Bond angles (°)	1.9
Ramachandran plot	
Most favored (%)	97.04
Allowed (%)	2.96

[†] CC_{1/2} represents the Pearson correlation coefficient for the highest resolution shell as described by Karplus & Diederichs (2012).

a key liability that would need to be addressed if this series were to be optimized for drug discovery. Structural insight might allow the series to be optimized for better cell permeability, and additionally the *N*-phenylbenzamides represent a novel chemical series with the potential to provide additional insight into the binding interactions that drive high affinity and species selectivity towards *Pf*DHODH. Here, we report the X-ray structure of DSM59 bound to *Pf*DHODH. The binding mode of DSM59 overlaps most closely with triazolopyrimidines (*e.g.* DSM267), but DSM59 forms unique interactions that have not been observed with previous compounds and which are likely to contribute to its high affinity for *Pf*DHODH.

2. Methods

2.1. Crystallization

N-terminally truncated *Pf*DHODH (amino acids 162–565) was expressed in *Escherichia coli* BL21 phage-resistant cells (Novagen) from plasmid pET-28b-TEV-*Pf*DHODH_{Δ384–413} as described previously (Booker *et al.*, 2010; Deng *et al.*, 2009). In addition to the N-terminal truncation that removed the membrane-spanning domain (residues 1–161), an extra loop truncation (amino acids 384–413) is also present in this construct because we previously found that this truncation significantly improved crystallization. *Pf*DHODH_{Δ384–413} was purified by Ni²⁺-agarose and gel-filtration chromatography as described previously (Deng *et al.*, 2009). Preliminary crystallization conditions for *Pf*DHODH_{Δ384–413}–DSM59 were found using the random crystallization screen The Cryos Suite.

Figure 2

Electron-density map for *Pf*DHODH–DSM59. The *F*_o – *F*_c map calculated before the model was refined and prior to building in the ligand is contoured in green (+3σ) and red (–3σ). The 2*F*_o – *F*_c map calculated after the model was refined contoured at 1σ is in blue. DSM59 is shown in pink and amino-acid residues are shown in yellow.

Crystallization took place using the hanging-drop vapor-diffusion method at 20°C. The crystallization drop consisted of equal volumes of reservoir solution and *Pf*DHODH $_{\Delta 384-413}$ (20 mg ml $^{-1}$) pre-equilibrated with 1 mM DSM59 and 2 mM dihydroorotate (DHO). The crystallization conditions (reservoir solution) were 0.16 M ammonium sulfate, 0.1 M sodium acetate pH 5.35, 13.5% PEG 4000, 24% glycerol, 10 mM DTT. Crystals typically grew in two weeks. *N*-(3,5-Dichlorophenyl)-2-methyl-3-nitrobenzamide (DSM59) was synthesized as described previously (Malmquist *et al.*, 2008)

2.2. Data collection and processing

Diffraction data were collected at 100 K on beamline 19ID at the Advanced Photon Source (APS) using an ADSC Q315 detector. The *Pf*DHODH $_{\Delta 384-413}$ –DSM59 crystal diffracted to 2.3 Å resolution and belonged to space group $P6_4$, with unit-cell parameters $a = b = 85.0$, $c = 137.7$ Å. A single molecule of *Pf*DHODH was found in the asymmetric unit with a 61% volume of bulk solvent. Diffraction data were integrated and

intensities were scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997).

2.3. Structure determination and refinement

Crystallographic phases for *Pf*DHODH $_{\Delta 384-413}$ –DSM59 were solved by molecular replacement with *Phaser* (McCoy, 2007) using the previously reported structure of *Pf*DHODH $_{\Delta 384-413}$ bound to DSM1 (PDB entry 3i65; Deng *et al.*, 2009) as a search model (bound ligands were removed from the model; Fig. 2 and Table 2). The structure was rebuilt with *Coot* (Emsley & Cowtan, 2004) and refined with *REFMAC* (Murshudov *et al.*, 2011). The *Pf*DHODH $_{\Delta 384-413}$ –DSM59 structure was refined to an R and R_{free} of 0.18 and 0.27, respectively. All residues (amino acids 161–383 and 414–565) were within the allowed region of the Ramachandran plot (Table 2). Water molecules (50) were added if the density was stronger than 3.4σ in the difference density map obtained with *ARP/wARP* (Lamzin & Wilson, 1993) and were manually removed if the density was weaker than 1σ in the $2F_o - F_c$

Figure 3

Structure of DSM59 bound to *Pf*DHODH. (a) Ribbon diagram showing the DSM59 binding site on *Pf*DHODH. DSM59 is displayed in pink, FMN in yellow and L-oro in turquoise; protein C atoms are in turquoise, O atoms in red, N atoms in blue, phosphate in orange, S atoms in yellow and chloride in green. (b) Stereoview of the DSM59 binding site showing amino-acid residues within a 4 Å shell. DSM59 is displayed in pink, FMN, L-oro and protein C atoms are in turquoise, O atoms in red, N atoms in blue, phosphate in orange, S atoms in yellow and chloride in green.

map obtained with *Coot*. *PyMOL* (Schrödinger) was used to generate all structure figures. The coordinates of the structure have been deposited in the PDB (PDB entry 4ryh).

3. Results and discussion

3.1. DSM59 inhibitor-binding site

*Pf*DHODH belongs to the β/α -barrel structural fold class and DSM59 binds in a site between the two N-terminal α -helices (starting at amino acid 162) and the body of the barrel domain (Fig. 3*a*). The binding site is adjacent to the flavin mononucleotide (FMN) cofactor and is largely hydrophobic in nature. As has been observed for other DHODH inhibitors, DSM59 is a competitive inhibitor of CoQ (Baldwin *et al.*, 2005), suggesting that this binding site overlaps with that of CoQ. However, no structural data are available for CoQ bound to DHODH from any species. The dichloroaniline group of the inhibitor is bound in an entirely hydrophobic pocket where it is in van der Waals contact with Met536,

Table 3

Comparison of *Pf*DHODH protein–inhibitor distances (Å) for representative inhibitors from different structural classes.

	DSM59	DSM267	Genz 667348
His185 ND1	N1, 2.6	N1, 2.7	N3, 3.0
Arg265 NE	O3 (NO ₂), 3.7	N4, 3.9	O2, 4.2
Arg265 NH1	O3, 5.7	N5, 2.8	O2, 2.8
Glu182 N	O1 (NO ₂), 3.1	F5, 3.5	NA
Val532 N	O1, 4.2	NA	NA
Glu535 C	Cl1, 3.4	NA	NA

Gly535, Leu531, Ile237, Cys233, Phe188 and Phe227 and where it forms edge-to-face stacking interactions with both Phe188 and Phe227 (Fig. 3*b*). One of the two Cl atoms is within a short distance of the backbone carbonyl of Gly535 (C–Cl distance of 3.4 Å) at just the optimal angle (90°) to the C=O axis to maximize a favorable binding interaction of the chlorine in a ‘halogen bond’ (Auffinger *et al.*, 2004) with the delocalized π electrons of the Gly535–Met536 amide. The bridging N atom forms a good hydrogen bond (2.6 Å) to ND1 of His185 (Fig. 3*b* and Table 3). The carbonyl of the amide group is not within hydrogen-bonding distance of any amino acid in the protein and its closest contact is with the backbone amide of Val532 (at a distance of 4.2 Å).

The nitrobenzyl group is bound between His185 and Arg265. As a consequence of steric interaction with the adjacent methyl group, the nitro group is twisted 46.4° out of the benzene plane. This is close to the observed nitro-group twist of 38.8° in the small-molecule structure of 1-chloro-2-methyl-3-nitrobenzene (Pearce & Tanski, 2011). As a consequence of this out-of-plane twist, the planar nitro group lies roughly parallel to and directly above the plane of the peptide backbone of Gly181–Glu182. The N and one O atom of the nitro group are only 3.1 Å above the plane of the Gly181–Glu182 backbone amide, with the dipole moments of the two groups approximately antiparallel. Such a geometrical arrangement of dipoles leads to a highly favorable stacking energy of interaction between the inhibitor and the DHODH backbone amide (Pearce & Tanski, 2011). The second O atom of the nitro group is 3.7 Å from the NE atom of Arg265; however, this residue is turned away from the inhibitor, prohibiting more extensive contacts between the two groups, and thus the Arg265–NO₂ interaction is primarily charge–charge in nature. The rotation of Arg265 away from the inhibitor pocket is required to accommodate the bulk of the nitro group, which extends further into the pocket towards Arg265 than either DSM267 or Genz 667348 (see below).

3.2. Binding-mode comparisons between *Pf*DHODH inhibitor-binding sites

The *Pf*DHODH inhibitor-binding site has two components: the hydrogen-bond site between Arg256 and His185 (and nearest to FMN) and the adjacent hydrophobic pocket that is lined with residues in part contributed by helices 1 (amino acids 162–176) and 2 (amino acids 181–194; Booker *et al.*, 2010; Deng *et al.*, 2009). The amino-acid residues nearest FMN show the least conformational flexibility and generally align closely

Figure 4
Inhibitor-binding site comparison of the binding modes of DSM59 (turquoise protein with DSM59 ligand in pink) and (a) DSM267 (purple) from the triazolopyrimidine chemical series and (b) Genz 667348 (purple) from the thiophene carboxamide chemical series. Selected amino-acid residues within 4 Å of the inhibitor-binding sites are displayed.

between all known structures, whereas the residues in the hydrophobic pocket have been observed in multiple conformations. The most striking structural difference between the various co-crystal structures is the position of Phe188, which is rotated nearly 180° between the DSM267 and Genz 667348 binding modes, leading to the formation of two distinct hydrophobic pockets projecting from the common hydrogen-bond site (Booker *et al.*, 2010; Deng *et al.*, 2009).

The binding mode of DSM59 was compared with that of DSM267 (r.m.s.d. = 0.4 Å) and Genz 667348 (r.m.s.d. = 0.6 Å) as all three compounds inhibit *Pf*DHODH with similar potency (Table 1). All inhibitors for which structural data are available occupy the hydrogen-bond site, making hydrogen-bond contacts with Arg265, His185 or both residues, as illustrated by comparison of the DSM59 binding pocket with those of DSM267 and Genz 667348 (Figs. 4*a* and 4*b*). All three compounds make close hydrogen-bond contacts with His185 (Figs. 4*a* and 4*b* and Table 3), although the position of His185 is rotated in each structure to allow optimal geometry for the individual interactions. However, unlike DSM267 and Genz 667348, DSM59 does not form a hydrogen-bond interaction with Arg265 because the guanidinium is rotated away from the ligand in order to accommodate the NO₂ group (Figs. 4*a* and 4*b* and Table 3). However, it is likely that an electrostatic interaction between Arg265 and the NO₂ group of DSM59 contributes to the binding energy, and thus interactions with Arg265 are likely to be important to high-affinity binding of all three inhibitors. The importance of the binding interactions with both His185 and Arg265 is supported by site-directed mutagenesis studies, where we found that mutation of either residue to Ala reduced the binding affinity of *Pf*DHODH for DSM59 and for several characterized triazolopyrimidines (Malmquist *et al.*, 2008; Deng *et al.*, 2009).

The DSM59 binding mode overlaps most closely with the triazolopyrimidine binding mode (*e.g.* DSM267) with respect to the hydrophobic site. In both structures Phe188 is in the 'down' position, such that the dichloroaniline of DSM59 and the trifluoromethylaniline of DSM267 are largely overlapping (Fig. 4*a*). In contrast Phe188 is in the 'up' position when bound to Genz 667348, and the dichloroaniline of DSM59 and the benzimidazole of Genz 667348 occupy completely different binding sites (Fig. 4*b*). The dichloroaniline of DSM59 is pushed deeper into the pocket than the aniline ring of DSM267, allowing accommodation of the *meta* chlorines on the ring. In contrast, *meta* substituents on the aniline ring were not well tolerated in the triazolopyrimidine series as these substituents are too close to the narrow opening to the aniline binding pocket, and this position was sterically restricted (Deng *et al.*, 2009). Much of the space occupied by the pyrimidine of triazolopyrimidine DSM267 is vacant in DSM59. This results in significant protein adjustments in helix 1 and in the orientation of the side chains of residues Leu172 and Leu176 and in the position of Phe188 (helix 2), although Phe188 repositions to a much lesser extent than observed in the comparison with the Genz 667348 binding mode (Fig. 4*a*). The position of Met536 is also reoriented since the trifluoromethyl group on the aniline of DSM267 would clash with the

position of Met536 in the DSM59 structure. Finally, significant differences in the position of helix 1 are also present between the DSM59 and Genz 667348 structures as the helix and projecting residues move to accommodate the alternative binding mode of Phe188 and the benzimidazole group of Genz 667348 (Fig. 4*b*).

4. Conclusions

The X-ray structure of DSM59 in complex with *Pf*DHODH shows that DSM59 binds in a similar pocket to the triazolopyrimidine class of *Pf*DHODH inhibitors but, owing to its different shape and properties, the configuration of a number of binding-site residues have moved relative to their positions in other co-inhibitor complexes. DSM59 makes an important energetically favorable stacking interaction of its nitro group with the exposed protein amide between Gly181 and Glu182, a salt-bridge interaction with Arg265, a good 'halogen bond' between one of the dichloroaniline chlorines and the carbonyl of Gly535, the canonical hydrogen bond to His185 seen in all DHODH-inhibitor complexes and a number of hydrophobic and aromatic stacking interactions through the dichloroaniline that are also likely to contribute significantly to its potent and selective binding. Both the stacking interaction with Glu181/Glu182 and the 'halogen bond' are novel interactions that have not been observed in other *Pf*DHODH-inhibitor complexes, and both are likely to contribute significant binding energy to the interaction. These data show that the flexibility of the *Pf*DHODH inhibitor-binding site allows it to form tight interactions with diverse structural classes of small molecules, enhancing its value as a drug target.

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