Two crystal structures of dihydrofolate reductase-thymidylate synthase from Cryptosporidium hominis reveal protein–ligand interactions including a structural basis for observed antifolate resistance

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Cryptosporidium hominis is a protozoan parasite that causes acute gastrointestinal illness. There are no effective therapies for cryptosporidiosis, highlighting the need for new drug-lead discovery. An analysis of the protein–ligand interactions in two crystal structures of dihydrofolate reductase-thymidylate synthase (DHFR-TS) from C. hominis, determined at 2.8 and 2.87 Å resolution, reveals that the interactions of residues Ile29, Thr58 and Cys113 in the active site of C. hominis DHFR provide a possible structural basis for the observed antifolate resistance. A comparison with the structure of human DHFR reveals active-site differences that may be exploited for the design of species-selective inhibitors.

1. Introduction

Cryptosporidium hominis is a water-borne protozoan parasite that infects the intestinal epithelium of many mammals, including humans. This highly infectious agent, recently placed on the Centers for Disease Control list of Class B bioterrorism agents, causes cryptosporidiosis, manifested as self-limiting diarrhea in immune-competent patients and massive chronic diarrhea in immune-deficient patients (Gue rant, 1997; Tzipori, 1998). Despite the threat of cryptosporidiosis infection, to date there are no clinically successful therapies.

Infections from closely related parasitic protozoa such as Plasmodium falciparum and Toxoplasma gondii have been treated with antifolates, inhibitors of dihydrofolate reductase (DHFR), an essential enzyme in the folate-biosynthetic pathway. DHFR catalyzes the reduction of dihydrofolate using NADPH as a cofactor. In protozoa, a single polypeptide chain codes for both DHFR and thymidylate synthase (TS), forming a bifunctional enzyme. Unfortunately, attempts to treat cryptosporidiosis with antifolates have been unsuccessful. The failure of these drugs has been observed both in vivo (Woods et al., 1996) and in vitro (Nelson & Rosowsky, 2001). Vazquez et al. (1996) found that the sequence of C. hominis DHFR (ChDHFR) naturally contains Ile29, Thr58 and Cys113 at the same positions as the antifolate resistance-conferring mutations Ile51, Thr108 and Ile164 in P. falciparum DHFR (PiDHFR). The incorporation of these residues in ChDHFR was hypothesized to play an important role in in vitro antifolate resistance.

Since the sequence of DHFR has diverged and the sequence of TS has remained largely conserved throughout evolution, it is easier to design parasite-specific DHFR inhibitors instead of TS inhibitors. Therefore, in this work discussion will focus on the ChDHFR domain. In order to design selective and potent ChDHFR inhibitors in future studies, it is necessary to understand ligand binding in ChDHFR including (i) the interactions of the protein with ligands, (ii) the structural effects of the C. hominis residues predicted to play a role in antifolate resistance and (iii) ligand-induced conformational changes. Towards that goal, an analysis of two crystal structures of the enzyme DHFR-TS from C. hominis is presented. In one structure (structure I), presented in this manuscript, the enzyme is bound to an antifolate inhibitor, 1843U89 (OSI Pharmaceuticals), in the DHFR and TS active sites, as well as NADPH in the DHFR active site and the TS
substrate deoxyuridine monophosphate (dUMP) in the TS active site. In the second structure (structure II), which details regarding the crystallographic data and refinement have previously been published, the enzyme is bound to the DHFR substrate dihydrofolate (DHF) and the cofactor NADPH in the DHFR active site and dUMP and an antifolate TS inhibitor, CB3717, in the TS active site. This structure formed the basis of previously reported research (O’Neill et al., 2003a,b) that established that there are two families of DHFR-TS structures from protozoa; analysis of the binding of DHF and NADPH will appear for the first time in this work. A comparison of the structure of ChDHFR-TS with structures of the wild-type and the antifolate-resistant PidDHFR-TS (Yuvaniyama et al., 2003) shows that the ChDHFR residues hypothesized to be important in antifolate resistance have the same effects on the active site as the mutated *P. falciparum* residues known to be important in causing resistance, suggesting equivalent structural effects that decrease the affinity of antifolates. Additionally, a comparison of the two ChDHFR-TS structures reveals no ligand-induced conformational changes in the DHFR active site. A comparison of ChDHFR-TS with human DHFR reveals sites in ChDHFR-TS that may be exploited for the design of parasite-selective inhibitors.

### 2. Materials and methods

#### 2.1. DHFR activity assays

Enzyme purification has been described previously (O’Neill et al., 2003a,b). DHFR activity assays were performed using a solution of 50 mM TES buffer pH 7.0, 1 mM EDTA, 75 μM 2-mercaptoethanol, 1% bovine serum albumin, 1 mM dihydrofolate (Eprova) and 100 μM NADPH. Enzyme concentrations were adjusted to give linear initial velocities.

### 2.2. Crystallization and data collection

Pure enzyme was concentrated to 6.5 mg ml⁻¹ and incubated with ligands on ice for 1 h. The final concentrations of the ligands (note that all ligands were dissolved in water with the exception of TMP, which was dissolved in DMSO) were as follows: 1 mM TMP and 2 mM each of NADPH, dUMP and 1843U89 (OSI Pharmaceuticals). Crystals were grown by hanging-drop vapor diffusion at room temperature and appeared in drops where the reservoir solution contained 100 mM Tris pH 8.0, 11% PEG 6K, 50 mM ammonium sulfate and 0.2 M lithium sulfate. Crystals were transferred to a drop of artificial mother liquid containing 15% ethylene glycol and then transferred to a drop of artificial mother liquid containing 25% ethylene glycol before being frozen in liquid nitrogen.

Diffraction data were collected at 100 K at Stanford Synchrotron Radiation Laboratory beamline 7-1. All data were integrated, averaged and scaled with the HKL package (Otwinowski, 1993) and converted to structure factors with TRUNCATE (Collaborative Computational Project, Number 4, 1994; see Table 1 for data statistics).

### 2.3. Structure solution

The structure of ChDHFR-TS bound to 1843U89, NADPH and dUMP was solved by difference Fourier techniques using structure II and refined using CNS (Brünger et al., 1998; Table 1). The R-factor and Rfree values, after simulated annealing and manually rebuilding residues surrounding the inhibitors, are 21.8 and 23.6%, respectively. The root-mean-square deviation of bonds is 0.008 Å and the r.m.s.d. for angles is 1.6°.

### 3. Results and discussion

#### 3.1. Crystallographic structure determination

Structures I and II were solved with diffraction amplitudes extending to 2.87 and 2.8 Å, respectively. The resolution of these structures allows a determination of the proximity of hydrogen-bond donors and acceptors, but detailed measurements of hydrogen bonds will not be included. The determination of structure II has been described previously (O’Neill et al., 2003a,b; Lilien et al., 2004); structure I was solved using difference Fourier methods and phases from structure II. Electron density for the ligands and residues 3–521

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**Table 1**

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<thead>
<tr>
<th>Data and refinement statistics for the structure of ChDHFR-TS/NADPH/1843U89/dUMP</th>
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† Rfree = ∑ ||Fobs|−|Fc|)/∑|Fobs| · Rfree statistics for a test set comprising 10% of the reflections

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**Figure 1**

Stereo figure of 2Fobs – Fc electron density (shown at 2σ contour) for the DHFR active site with the inhibitor 1843U89 and NADPH shown in green.
is well defined (Fig. 1). In the DHFR active site, omit map calculations clearly reveal electron density for 1843U89 in structure I and DHF in structure II, despite the fact that the protein–ligand crystallization mix included the known DHFR inhibitors trimethoprim and methotrexate, respectively. The presence of 1843U89 may be explained by results from a ChDHFR assay showing that 1843U89 exhibits a 50% inhibition (IC$_{50}$) value of 5 μM, threefold tighter binding than trimethoprim (IC$_{50}$ = 14 μM). The presence of DHF may be explained by the fact that the protein was immediately crystallized after 2 mM DHF elution from the methotrexate agarose column.

ChDHFR-TS assembles as a dimer. In the asymmetric unit, one dimer is coincident with the crystallographic twofold axis and two dimers are located away from the axis, yielding five monomers per asymmetric unit. The quality of the electron density in all five monomers is essentially identical. The protein monomers were refined using non-crystallographic symmetry restraints.

3.2. **Overall description of the DHFR-TS structure**

ChDHFR-TS is a bifunctional protein organized with the N-terminal DHFR domain separated from the C-terminal TS domain by a 59-residue linker domain (Fig. 2). After folding the DHFR domain (1–175), residues from the linker domain (176–206) cross from one monomer of the dimer to the other and form a helix, called the donated helix, which packs against the opposite DHFR active site. The interactions of the donated helix with the opposite monomer have been described in detail in a previous publication (O’Neil et al., 2003a,b). The linker then crosses back to the originating monomer, forming a ninth strand in the β-sheet of DHFR, and then forms the TS domain (residues 207–521; O’Neil et al., 2003a,b). In the crystal structures described here, ChDHFR has features that define the closed conformation of *P. carinii* DHFR (PeDHFR; Cody et al., 1999), suggesting that ChDHFR is in the closed conformation.

3.3. **Ligand binding in ChDHFR-TS**

NADPH is bound to ChDHFR in an extended conformation (Figs. 3a and 3b). There are several hydrophobic interactions between NADPH and ChDHFR, including Ile75 and Ile118 with the adenosine, and Leu25, Ile19, Thr58, Tyr119, Gly115 and the pteridine ring of dihydrofolate or the benzoquinazoline ring of 1843U89 with the nicotinamide ring. Several potential hydrogen bonds are also apparent: Ser76, Ser77 and Arg56 with the O atoms of the adenosyl phosphate, Thr58 and Ser117 with the O atoms of the bridge phosphate and the backbone of Ala11 with the amino and keto oxygen groups of the nicotinamide ring.

The antifolate 1843U89 (OSI Pharmaceuticals), originally developed as an anticancer TS inhibitor, is bound in the DHFR active site of structure I (Fig. 3a). One hydrogen bond is formed from the benzoquinazoline ring to Asp32 and the methyl group on that ring is buried in a small hydrophobic pocket comprised of Thr134, Ala11 and Val9. Phe36, Leu25 and Ile62 form other hydrophobic contacts with the benzoquinazoline ring. The isoindolinone moiety is bound in a highly hydrophobic pocket comprised of Leu67, Ile62, Met54, Leu33, Phe36 and Leu25. The folate tail makes two potential hydrogen bonds with Arg70 and one with an ordered water molecule.

Interestingly, 1843U89 is bound in both the DHFR and TS active sites of structure I. It is noteworthy that both sites include a predominance of hydrophobic interactions. In fact, there
are no potential hydrogen bonds between 1843U89 and \textit{C. hominis} TS and only four in ChDHFR, despite the number of hydrogen-bond donors and acceptors in 1843U89.

Dihydrofolate is bound to ChDHFR in the same conformation as in the human enzyme (PDB code 1dfr; Oefner et al., 1988; Fig. 3b). The pteridine ring forms three hydrogen bonds with the protein: one from the amino group to Thr134, another from the ring NH to the carboxylate of Asp32 and a third from the amino group to Asp32. The pteridine ring also has extensive van der Waals contacts with Phe36, Val9 and the nicotinamide ring. The \textit{para}-aminobenzoic acid (pABA) binds in the same location as the isoidolamine ring of 1843U89 and makes many of the same hydrophobic interactions with the protein. There are three potential hydrogen bonds formed between the glutamate tail of dihydrofolate and the protein: two bonds with Arg70 and a unique hydrogen bond relative to other sites with the side chain of Ser37.

3.4. Structural effects of residues predicted to be important in antifolate resistance

Using a comparison of the structures of wild-type and resistant PdDHFR-DS, YuYanima and coworkers determined many of the structural effects of the PdDHFR mutations known to cause antifolate resistance (Yuyanima et al., 2003). Vasquez et al. (1996) used a sequence alignment (Fig. 4) to show that residues Ile29, Thr58 and Cys113 are at the same positions as the mutations Ile51, Asn108 and Leu164 in resistant PdDHFR. A comparison of ChDHFR with the structures of wild-type PdDHFR and the quadruple mutant (Asn51Ile, Cys59Arg, Ser60Asn, Ile164Leu) PdDHFR (YuYanima et al., 2003) (Fig. 5) reveals that ChDHFR residues Ile29, Thr58 and Cys113 have equivalent structural perturbations on the active site.

Wild-type PdDHFR contains Asn51, which forms a hydrogen bond with the backbone amide of the active-site Asn, which in turn forms hydrogen bonds with the diaminopyrimidines of most antifolate inhibitors. Ile29 (ChDHFR) and the resistance mutation Ile51 (PdDHFR) superimpose; both lose the hydrogen bond with the active-site Asn, creating a backbone shift at the active site. Thr58 in ChDHFR superimposes with the PdDHFR mutation Asn108. Asn108 creates steric interactions with the nicotinamide ring of NADPH and the \textit{para}-chlorophenyl group of pyrimethamine (Yuyanima et al., 2003). The same interactions are apparent for Thr58 in ChDHFR. Finally, in PdDHFR the Ile164Leu mutation causes an additional sevenfold reduction in binding to pyrimethamine (Sirawaraporn et al., 1997), presumably owing to reduced van der Waals contacts. In ChDHFR, Cys113 exhibits an even greater reduction in van der Waals contacts (Fig. 5), possibly severely decreasing antifolate affinity.

Evidence from field isolates (Basc et al., 1996) and in vitro experiments (Sirawaraporn et al., 1997) shows that the appearance of multiple mutations in resistant strains of \textit{Plasmodium} has a synergistic effect in decreasing the affinity of antifolate inhibitors. For example, the Ser108Thr mutation by itself does not lead to a high degree of resistance, but this mutation in combination with Ala16Thr leads to high levels of cycloguanil resistance (Foote et al., 1990; Peterson et al., 1990). The same argument may apply to \textit{C. hominis}: a single residue may not contribute more than a slight increase in the binding constant of an inhibitor, but the accumulation of multiple residues may cause a significant loss of affinity. In ChDHFR, the decreased van der Waals surface of Cys131, the steric interactions of Thr58 and the loss of a key hydrogen bond from Ile29 may together lead to a pronounced decrease in antifolate binding.

3.5. Ligand-induced conformational changes

A comparison of ChDHFR bound to DHF and 1843U89 surprisingly reveals that there are few conformational changes in the protein induced by the differences between DHF and 1843U89. The benzoquinazoline ring of 1843U89 fits into the same space as the CH\textsubscript{2}N bridge of DHF. The isoidolamine appears to be accommodated in a greater volume of the hydrophobic pocket and forms contacts with Ile62, Leu67, Leu33 and Phe36. In comparison, the pABA ring of DHF contacts the same residues, with the exception of Leu67.

3.6. Selectivity: comparison with human DHFR

A superposition of the structures of ChDHFR with DHFR from human (PDB code 1dfr; Oefner et al., 1988), \textit{P. carinii} (PBD code 2ed2; Cody et al., 1999) and \textit{Escherichia coli} (PDB code 1rb5; Sawaya & Kraut, 1997) shows strong conservation of the overall conformation of DHFR, but also reveals a significant difference between ChDHFR, PdDHFR and human DHFR. Human DHFR has a loop (residues 63–65 and sequence Pro-Glu-Lys-Asn, PEKN) near the exit to the active site; this loop is deleted in ChDHFR (Figs. 4 and 6). The conformation of the PEKN loop and the initial proline residue is conserved in most other eukaryotic DHFR species. The proline in the human DHFR structure (Oefner et al., 1988) is only 4 Å from the pABA ring of dihydrofolate and forms van der Waals contacts with the ring.

Two other active-site residues differ between ChDHFR and human DHFR: Val9 and Leu33 in ChDHFR are Ile7 and Phe31, respectively, in


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human DHFR. The valine substitution has reduced van der Waals contact with the pteridine ring in structure II. The substitution of Leu33 relative to Phe31 in the human enzyme has been implicated in the selectivity of trimethoprim for parasitic and bacterial species over human (Kuyper et al., 1985; Roth et al., 1987).

In summary, the two structures of ChDHFR-TS reveal the interactions of residues in the DHFR domain with different ligands, show that the structural effects of the residues implicated in antifolate resistance are similar to the structural effects of the mutations found in resistant strains of *P. falciparum* and also reveal key structural differences with human DHFR. The information discovered here will be valuable in the design of potent and selective ChDHFR inhibitors.

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**References**


