Kinetic and Structural Analysis for Potent Antifolate Inhibition of *Pneumocystis jirovecii*, *Pneumocystis carinii*, and Human Dihydrofolate Reductases and Their Active-Site Variants

Vivian Cody,^{a,b} Jim Pace,^a Sherry F. Queener,^c Ona O. Adair,^d Aleem Gangjee^d

Structural Biology Department, Hauptman Woodward Medical Research Institute, Buffalo, New York, USA^a; Structural Biology Department, School of Medicine and Biological Sciences, State University of New York at Buffalo, Buffalo, New York, USA^b; Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana, USA^c; Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, Pittsburgh, Pennsylvania, USA^d

A major concern of immunocompromised patients, in particular those with AIDS, is susceptibility to infection caused by opportunistic pathogens such as *Pneumocystis jirovecii*, which is a leading cause of pneumonia in immunocompromised patients. We report the first kinetic and structural data for 2,4-diamino-6-[(2',5'-dichloro anilino)methyl]pyrido[2,3-*d*]pyrimidine (OAAG324), a potent inhibitor of dihydrofolate reductase (DHFR) from *P. jirovecii* (pjDHFR), and also for trimethoprim (TMP) and methotrexate (MTX) with pjDHFR, *Pneumocystis carinii* DHFR (pcDHFR), and human DHFR (hDHFR). OAAG324 shows a 9.0-fold selectivity for pjDHFR (K_i , 2.7 nM) compared to its selectivity for hDHFR (K_i , 24.4 nM), whereas there is only a 2.3-fold selectivity for pcDHFR (K_i , 6.3 nM). In order to understand the determinants of inhibitory potency, active-site mutations of pj-, pc-, and hDHFR were explored to make these enzymes more like each other. The most unexpected observations were that the variant pcDHFR forms with K37Q and K37Q/F69N mutations, which made the enzyme more like the human form, also made these enzymes more sensitive to the inhibitory activity of OAAG324, with K_i values of 0.26 and 0.71 nM, respectively. A similar gain in sensitivity was also observed for the hDHFR N64F variant, which showed a lower K_i value (0.58 nM) than native hDHFR, pcDHFR, or pjDHFR. Structural data are reported for complexes of OAAG324 with hDHFR and its Q35K and Q35S/N64F variants and for the complex of the K37S/F69N variant of pcDHFR with TMP. These results provide useful insight into the role of these residues in the optimization of highly selective inhibitors of DHFR against the opportunistic pathogen *P. jirovecii*.

Opportunistic organisms in the genus *Pneumocystis* are atypical fungi that reside in the lungs of mammals and can cause a lethal pneumonia in immunocompromised hosts (1, 2). *Pneumocystis* pneumonia (PCP), caused by *Pneumocystis jirovecii*, is one of the most-prevalent opportunistic infections in patients with HIV-AIDS (1), and more recently, its prevalence has become a major concern among non-HIV patients (3, 4). Current treatment of PCP combines the antifolates sulfamethoxazole (SMX) and trimethoprim (TMP) (Fig. 1): SMX targets dihydropteroate synthase (DHPS), and TMP targets dihydrofolate reductase (DHFR). Although TMP-SMX is still the most effective first-line therapy, more than one-third of patients experience dose-limiting toxicity (1–6). Failure of therapy or prophylaxis with TMP is common, but attempts to link these failures to variants of *P. jirovecii* dihydrofolate reductase (pjDHFR) that lead to alternative forms of the enzyme have been inconclusive (7, 8). To date, most structural and drug design efforts have focused on the DHFR from *Pneumocystis carinii* (pcDHFR), the organism originally isolated from rat lungs (9–12). Recent observations show differences in drug sensitivity between pc- and pjDHFR that suggest designed optimization of antifolates would more selectively target pjDHFR (13).

Sequence alignment of pjDHFR, the form of the enzyme found in the human pathogen *P. jirovecii*, and pcDHFR, from the rat pathogen *P. carinii*, reveals 79 amino acid differences between the two 206-residue enzymes, with a number of changes among their active-site residues (7) (Table 1 and Fig. 2). To understand the role of active-site residues that modulate the drug sensitivity of DHFR, attention was focused on those sites that had the most diversity among the three species of interest, i.e., positions 23/20, 33/31, 37, 35, and 69/64. Of these, position 69/64 seemed a top priority for

exploration because experimental compounds showed strong interactions with the phenylalanine at position 69 in pcDHFR (14). In addition, prior studies indicated that position 37/35 strongly influenced the sensitivity of pcDHFR to TMP (13). For these reasons, those residues at positions 35 and 64 of hDHFR and at positions 37 and 69 in both pcDHFR and pjDHFR were mutated to reflect the 24 combinations of single and double variants to make the active sites more like each another. As illustrated in Table 1, these residues are Gln35 and Asn64 in hDHFR, which correspond to Lys37 and Phe69 in pcDHFR and to Ser37 and Ser69 in pjDHFR. Similarly, double variants were created as a cross between the active sites of two different DHFR species.

The numbering used throughout this paper for the human DHFR sequence is based on the first position being Val1, rather than Met1 as observed in the gene sequence listing. This numbering scheme has been used in previous publications of the kinetic and structural data for hDHFR and is being used here for continuity.

Received 24 January 2013 Returned for modification 20 February 2013

Accepted 23 March 2013

Published ahead of print 1 April 2013

Address correspondence to Sherry F. Queener, queenes@iupui.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00172-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00172-13

FIG 1 Schematics of antifolates under study. 1, OAAG324; 2, trichlorophenyl analogue.

Efforts to design more-selective antifolates that target opportunistic pathogens, including those from the genus *Pneumocystis*, have been the focus of several studies (9–12). Analysis of inhibition data for 2,4-diamino-5-pyrido[2,3-*d*] pyrimidine analogues against pcDHFR revealed that dimethoxy- or dichlorophenyl substitutions produced advantageous electron-withdrawing or electron-donating effects. Of this series, 2,4-diamino-6-[(2',5'-dichloroanilino)methyl] pyrido[2,3-*d*]pyrimidine (OAAG324) was found to be the most promising against pcDHFR, with a selectivity ratio (rat liver DHFR/pcDHFR) of 15.7 based on 50% inhibitory concentrations (IC_{50} s) measured at 90 μ M dihydrofolic acid (DHFA). The trichlorophenyl analogue (Fig. 1) was also a potent inhibitor, with a selectivity ratio of 13.0 (15).

The data reported herein are the first measurements of K_i values of OAAG324 toward pj-, pc-, and hDHFR and the 24 single and double active-site variants with substitutions at positions 37 and 69 (pjDHFR numbering) that make pj-, pc-, and hDHFR more like each other. These data reveal that OAAG324 is a more-potent inhibitor of pjDHFR than is TMP. We also report the first crystal structures of wild-type hDHFR and its Q35K and Q35S/N64F variants with OAAG324 and of the K37Q variant of pcDHFR with OAAG324. Additionally, the structure of the K37S/F69N variant of pcDHFR as a ternary complex with TMP and NADPH is also reported. These data are compared with hDHFR TMP complexes (PDB 2w3a) (16) and with the TMP complexes with the Q35K/N64F and Q35S/N64F variants of hDHFR (17), as well as the previously determined structure of the pcDHFR ternary complex with NADPH and TMP (18).

MATERIALS AND METHODS

Expression and purification of DHFR. The expression and purification of recombinant pj-, pc-, and hDHFR and its active-site variants were carried out as previously described (13). The cDNA for pj- or pcDHFR was transfected into *Escherichia coli* Rosetta Gami B (DE3) competent

TABLE 1 Comparison of active-site residues for pj-, pc-, and hDHFR^a

Origin of DHFR	Active-site residues
<i>P. jirovecii</i>	Gln23, Asp24, Asp32, Met33, Phe35, Ser37 , Leu62, Ser69 , Ala131
<i>P. carinii</i>	Asn23, Ser24, Glu32, Ile33, Tyr35, Lys37 , Ile62, Phe69 , Ala131
Human	Gly20, Asp21, Glu30, Phe31, Tyr33, Gln35 , Ile60, Asn64 , Glu123

^a Data for variant forms of these DHFR are reported for the residues labeled in bold. See Fig. 2 for the locations of these residues.

FIG 2 hDHFR is shown in green, with the active-site positions that differ among h-, pc-, and pjDHFR sequences (Table 1) highlighted in yellow. The inhibitor OAAG324 is shown in red. Drawn with PyMOL (MacPyMOL; <http://www.pymol.org>).

cells with pET-SUMO-DHFR plasmid for expression. Mutations were introduced into the cDNA for pj- and pcDHFR, and the entire coding sequence was verified by Roswell Park Cancer Center (Buffalo, NY). DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and used without further purification. Mutagenesis was performed by using the QuikChange site-directed mutagenesis kit (Stratagene). The SUMO fusion protein has the His tag for Ni column purification. A two-step purification protocol using a Ni-chelating immobilized-metal affinity chromatography (IMAC) for removal of the His-tagged protein, followed by Ulp1 protease cleavage and passage over a second IMAC column, resulted in a yield of 20 to 30 mg of purified enzyme from 1 liter of culture. The removal of the tag by the SUMO protease Ulp1 leaves the native protein sequence.

Kinetic analysis. Standard DHFR assays were conducted at 37°C with continuous recording of change of absorbance at 340 nm. The assay contained 41 mM sodium phosphate buffer at pH 7.4, 8.9 mM 2-mercaptoethanol, 150 mM KCl, and saturating concentrations of NADPH (117 μ M). DHFA was used at two to four different concentrations ranging from 9 to 90 μ M; full inhibition curves at each separate DHFA concentration allowed robust calculation of K_i , as noted below. The initial linear rates of enzyme activity were measured; rates were linear under standard conditions for 1 to 5 min, depending upon the enzyme being assayed. The activity was linearly related to the protein concentration under these conditions of assay.

K_m values were determined by holding either the substrate or cofactor at a constant, saturating concentration and varying the other over a range of concentrations. K_m values were determined by fitting the data to the Michaelis-Menten equation with or without substrate inhibition, using nonlinear regression methods to select the best statistical fit (Prism 4.0). The value of k_{cat} was determined from the V_{max} value and the protein concentration ($k_{cat} = V_{max}/[E_{tot}]$, where E_{tot} is the total concentration of DHFR in the assay). Competitive inhibition was confirmed for each wild-type and variant enzyme form by demonstrating that, in the presence of differing concentrations of inhibitor, the V_{max} in Michaelis-Menten plots did not change but the apparent K_m increased with increasing inhibitor concentration (13).

K_i values were determined by measuring the inhibition of the reaction at two or more concentrations of the substrate, dihydrofolic acid (DHFA). For the competitive inhibitors TMP and OAAG324 in this study, K_i could be calculated from the equation $K_i = IC_{50}/(1 + S/K_m)$, where IC_{50} is 50% inhibitory concentration and S is the concentration of DHFA.

Data for standard compounds were remeasured as controls for validation of our assays, and all analyses are based upon direct spectrophotometric measurements of the concentration of DHFA in the assays. Statis-

tical comparisons were performed with InStat 3.0, using the conservative nonparametric analysis of variance (ANOVA) because variances among groups were not always equal.

Crystallization. (i) **hDHFR-OAAG324, hDHFR-Q35S/N64F-OAAG324, and hDHFR-Q35K-NADPH-OAAG324.** Different protein concentrations (6.6 to 9.8 mg/ml) of the hDHFR samples were dissolved in 100 mM K_2HPO_4 , pH 6.9, 30% saturated $(NH_4)_2SO_4$ and set up in 10- μ l hanging drops. The protein was incubated with a 10:1 molar excess of NADPH and inhibitor OAAG324 over ice for 1 h prior to crystallization. Crystals were grown on glass coverslips at 14°C by vapor diffusion. The reservoir contained 100 mM K_2HPO_4 , pH 6.9, 60 to 64% saturated $(NH_4)_2SO_4$, 3% (vol/vol) ethyl alcohol (EtOH). All hDHFR crystals were cryoprotected with Paratone-N oil (Hampton Research, Aliso Viejo, CA).

(ii) **pcDHFR-K37Q-OAAG324.** Crystals were grown at 4°C by vapor diffusion hanging drops. The protein concentration was 20.4 mg/ml in 20 mM MES (morpholineethanesulfonic acid), pH 6.0, 100 mM KCl in 10- μ l drops.

(iii) **pcDHFR-K37S/F69N-NADPH-TMP.** A protein concentration of 10.9 mg/ml of the pcDHFR mutant was dissolved in 10 mM MES, pH 6.0, and 100 mM KCl. These proteins were incubated with a 10:1 molar excess of NADPH and inhibitor OAAG324 or TMP over ice for 1 h prior to crystallization. Crystals were grown on glass coverslips at 14°C by vapor diffusion. The reservoir contained 33 to 36% polyethylene glycol (PEG 2K), 50 mM MES, pH 6.0, and 100 mM KCl. Crystals were cryoprotected in 24% glycerol prior to mounting.

Data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) facility using the remote access protocol (19–21) on beamline 9-2 for the hDHFR binary complex with OAAG324, the hDHFR Q35S/N64F double mutant binary complex with OAAG324, and the pcDHFR K37S/F69N double mutant ternary complex with NADPH and TMP; data for the pcDHFR K37Q mutant ternary complex with OAAG324 was collected on beamline 9-1 at SSRL, and data for the hDHFR Q35K mutant ternary complex with OAAG324 and NADPH was collected on a Rigaku RaxisIVc imaging plate system with MaxFlux optics. Diffraction statistics are shown in Table S1 in the supplemental material for all complexes.

Structure determination. The structures of the hDHFR complexes were solved by molecular replacement methods using the coordinates for human DHFR (PDB 1U72) (22) in the program Molref (23), while those for pcDHFR used the coordinates for pcDHFR (PDB 1DYR) (18). Inspection of the resulting difference electron density maps was performed using the program COOT (24) running on a Mac G5 workstation and revealed binary inhibitor complexes for hDHFR-OAAG324 and the Q35S/N64F double variant-OAAG324 and a ternary complex for the Q35K single variant hDHFR complex with NADPH and OAAG324, as well as an inhibitor complex for pcDHFR-TMP (see Table S1 in the supplemental material). Data to a 2.2-Å resolution were collected at SSRL beamline 9-1 for a ternary complex of OAAG324 and NADPH with the K37Q variant of pcDHFR (see Table S2). The crystal was a thin plate with high mosaicity. Unlike the majority of other pcDHFR crystal structures reported, this crystal showed an orthorhombic lattice, $P2_12_12_1$. Difference electron density maps revealed the positions of molecule 1 and NADPH; however, difficulties with the refinement, which had high B values (high R values: $R/R_f = 26/35\%$), suggested the crystal was disordered or twinned.

To monitor the refinement, a random subset of all reflections was set aside for the calculation of R_{free} (5%). The model for the inhibitor OAAG324 and its parameter files were prepared using the Dundee PROGR2 Server website (<http://davapc1.bioch.dundee.ac.uk/programs/prodrg>) (25). The final cycles of refinement were carried out using the program Refmac5 in the CCP4 suite of programs (23). The Ramachandran conformational parameters from the last cycle of refinement were generated by RAMPAGE (26) and showed that the majority of residues in all complexes have the most favored conformation and none are in the disallowed regions (see Table S1 in the supplemental material). Molecular diagrams were made using PyMOL (MacPyMOL; <http://www.pymol.org>).

Protein structure accession numbers. Coordinates and crystallographic structure factors for human wild-type and variant DHFR inhibitor complexes and for the double variant pcDHFR TMP complex have been deposited in the Protein Data Bank under the accession codes 4g95 (hDHFR-OAAG324), 3L3R (hDHFR-Q35K-OAAG324), 3aof (hDHFR-Q35S/N64F-OAAG324), and 4g87 (pcDHFR-K37S/F69N-TMP).

RESULTS

Steady-state kinetic parameters. Kinetic parameters for the pj-, pc-, and hDHFR active-site variants were determined in order to assess how much influence the two active-site positions selected for study had on the catalytic properties of the enzyme forms (Table 2). Both pjDHFR and pcDHFR have statistically significantly higher K_m values for DHFA and for NADPH than the human enzyme. Substitutions at position 35 (or 37) or at 64 (or 69) had no statistically significant effects on the K_m for NADPH within any DHFR form, but several substitutions in pcDHFR elevated the K_m for DHFA above the wild-type value of 3.5 μ M (Table 2). The tendency to elevation of the K_m for DHFA with substitutions at these two sites was also seen with pjDHFR, but only the S69F and S37K/S69F substitutions had statistically significant effects.

The catalytic rate constants for hDHFR and pjDHFR were similar (26 and 36 s^{-1} , respectively), but the value of k_{cat} for pcDHFR was consistently significantly higher (235 s^{-1}). Interestingly, the introduction of an amino acid from either the hDHFR or pjDHFR active site at position 37 (K37Q or K37S) dropped the k_{cat} into the range for wild-type pjDHFR and hDHFR. Overall, the effect of substitutions at positions 37 and 69 was to impair the catalytic efficiency of the pcDHFR (Table 2, DHFA k_{cat}/K_m). Less effect was seen with substitutions in pjDHFR, but hDHFR substitutions at the two sites produced either no change or slight increases in catalytic efficiency.

Inhibition of DHFR variants by antifolates. K_i values for native and for variant DHFR enzymes were determined for OAAG324 and TMP in order to assess the role of amino acid substitutions at positions 37 and 69 in controlling the binding of substrate analogs in the active site of the enzymes (Table 3). The selectivity of OAAG324 toward pjDHFR relative to its selectivity toward hDHFR was confirmed by these analyses; the ratio of the K_i values for hDHFR/pjDHFR was 9.0. OAAG324 was also selective for pjDHFR relative to its selectivity for pcDHFR: the K_i ratio for pcDHFR/pjDHFR was 2.3. The results also confirmed the greater potency of TMP toward pjDHFR (K_i , 35 nM) than toward pcDHFR (K_i , 305 nM), as well as a higher selectivity of TMP for pjDHFR than for pcDHFR relative to hDHFR: K_i hDHFR/ K_i pjDHFR was 117, and K_i hDHFR/ K_i pcDHFR was 13.4.

Variants that convert the pcDHFR enzyme to a more pjDHFR-like active site lowered the pcDHFR K_i for OAAG324 from 6.3 nM toward the pjDHFR value (2.7 nM), e.g., the K37S (0.8 nM) and K37S/F69S (1.42 nM) variants. In contrast, the K_i value of the single variant F69S (8.31 nM) was not significantly different from that of the wild-type enzyme (6.3 nM) (Table 3). However, the largest gain of function was observed for those variants that convert pcDHFR to a more human-like active site, with the K_i values for the K37Q single variant (0.26 nM) and the K37Q/F69N double variant (0.71 nM) being significantly different from those for pcDHFR (6.3 nM) or pjDHFR (2.7 nM) (Table 3).

Variants of the human DHFR enzyme that convert it to a more pjDHFR-like active site lowered the hDHFR K_i for OAAG324

TABLE 2 Kinetic constants for substrates and cofactor for pj-, pc-, and hDHFR and its active-site variants that reflect human-to-*Pneumocystis* changes

Enzyme description	DHFR form	Source of amino acid at position:		Mean $K_m \pm \text{SEM}$ (μM) of ^a :		Mean $k_{\text{cat}} \pm \text{SEM}$ (s^{-1}) ^a	k_{cat}/K_m for DHFA ($\text{s}^{-1} \mu\text{M}^{-1}$)
		37 (or 35 in hDHFR)	69 (or 64 in hDHFR)	DHFA	NADPH		
Wild type	hDHFR	hDHFR	hDHFR	0.7 ± 0.1 (8)	4.5 ± 1.2 (3)	26 ± 2 (9)	37
Q35K	hDHFR	pcDHFR	hDHFR	0.5 ± 0.2 (4)	12.5 ± 5.8 (3)	$39 \pm 2^*$ (6)	74
N64F	hDHFR	hDHFR	pcDHFR	0.8 ± 0.2 (5)	7.5 ± 3.1 (3)	28 ± 3 (3)	36
Q35K/N64F	hDHFR	pcDHFR	pcDHFR	0.4 ± 0.05 (8)	5.7 ± 1.1 (3)	$43 \pm 3^*$ (4)	106
Q35S	hDHFR	pjDHFR	hDHFR	0.7 ± 0.2 (4)	3.8 ± 1.4 (3)	24 ± 1 (5)	37
N64S	hDHFR	hDHFR	pjDHFR	0.9 ± 0.3 (3)	6.3	36 ± 5 (4)	41
Q35S/N64S	hDHFR	pjDHFR	pjDHFR	0.4 ± 0.05 (2)	5.3 ± 1.5 (3)	23 ± 2 (4)	54
Q35K/N64S	hDHFR	pcDHFR	pjDHFR	0.7 ± 0.2 (6)	6.6 ± 1.8 (3)	36 ± 0.4 (5)	51
Q35S/N64F	hDHFR	pjDHFR	pcDHFR	0.7 ± 0.2 (5)	5.6 ± 0.5 (3)	21 ± 2 (4)	32
Wild type	pcDHFR	pcDHFR	pcDHFR	3.5 ± 0.3 (10)	23 ± 1 (2)	235 ± 12 (6)	67
K37Q	pcDHFR	hDHFR	pcDHFR	6.6 ± 0.6 (9)	7.2 ± 0.4 (7)	$38 \pm 4^\dagger$ (10)	6
F69N	pcDHFR	pcDHFR	hDHFR	Not available	16.3	Not available	
K37Q/F69N	pcDHFR	hDHFR	hDHFR	$10.7 \pm 0.7^\dagger$ (8)	16.3 ± 1 (5)	223 ± 22 (4)	21
K37S	pcDHFR	pjDHFR	pcDHFR	$10.1 \pm 1.2^\dagger$ (10)	6.6 ± 1.8 (2)	$45 \pm 2^\dagger$ (8)	4
F69S	pcDHFR	pcDHFR	pjDHFR	7.1 ± 0.5 (11)	19.2 ± 1 (3)	204 ± 13 (11)	29
K37S/F69S	pcDHFR	pjDHFR	pjDHFR	$11.6 \pm 0.7^\dagger$ (12)	7.0 ± 0.4 (3)	157 ± 7 (9)	14
K37Q/F69S	pcDHFR	hDHFR	pjDHFR	$18.5 \pm 0.7^\dagger$ (11)	10.6 ± 0.3 (2)	158 ± 4 (11)	9
K37S/F69N	pcDHFR	pjDHFR	hDHFR	$9.7 \pm 0.5^\dagger$ (12)	16.7 ± 0.3 (2)	190 ± 15 (11)	20
Wild type	pjDHFR	pjDHFR	pjDHFR	2.0 ± 0.2 (17)	18.9 ± 1.5 (4)	36 ± 2 (20)	18
S37Q	pjDHFR	hDHFR	pjDHFR	3.5 ± 0.6 (5)	11.8 ± 1.6 (6)	51 ± 7 (7)	15
S69N	pjDHFR	pjDHFR	hDHFR	3.4 ± 0.6 (4)	25.2 ± 3.4 (3)	47 ± 7 (5)	14
S37Q/S69N	pjDHFR	hDHFR	hDHFR	3.8 ± 0.6 (6)	17.3 ± 0.4 (4)	$95 \pm 5^\P$ (5)	25
S37K	pjDHFR	pcDHFR	pjDHFR	Not available	Not available	Not available	
S69F	pjDHFR	pjDHFR	pcDHFR	$4.8 \pm 1.2^\P$ (5)	16.2 ± 0.2 (2)	$83 \pm 10^\P$ (5)	17
S37K/S69F	pjDHFR	pcDHFR	pcDHFR	$3.2 \pm 0.2^\P$ (14)	14.9 ± 0.7 (3)	57 ± 3 (11)	18
S37K/S69N	pjDHFR	pcDHFR	hDHFR	2.4 ± 0.4 (9)	86.4 ± 0.2 (2)	$78 \pm 9^\P$ (9)	33
S37Q/S69F	pjDHFR	hDHFR	pcDHFR	3 ± 0.3 (4)	27.5 ± 1.4 (3)	26 ± 2 (4)	9

^a The number of replicates in the determination is shown in parentheses. Those enzymes marked “Not available” were expressed but were either insoluble or catalytically inactive and thus could not be studied. Statistical comparisons were performed with InStat 2.03, using conservative nonparametric ANOVA. All values statistically significantly different from the respective control are shown in bold. *, Statistically different from wild-type hDHFR; †, statistically different from wild-type pcDHFR; ‡, statistically different from wild-type pjDHFR.

from 24.4 nM toward the pjDHFR value (2.7 nM) for the Q35S and N64S single variants (8.4 and 11.4 nM, respectively), but the difference was statistically significant only for the Q35S/N64S double variant (K_i , 2.5 nM). Variants converting human enzyme active-site residues to pcDHFR residues (Q35K, N64F, and Q35K/N64F) showed the most profound effects on binding of OAAG324 at the N64F site (K_i , 0.58 nM). The influence of the N64F substitution was confirmed by the fact that the Q35S/N64F cross double variant had a K_i value of 1.6 nM, well below the value of 9.6 nM determined for the Q35K/N64S cross double variant (Table 3).

Similarly, these data revealed that substitutions in pjDHFR to make it more like pcDHFR (i.e., S39F and S37K/S69F) (Table 3) had little effect on K_i , as the values were increased only slightly above wild-type values. The S37Q and S37Q/S69N variants that make pjDHFR more like hDHFR tended to have K_i values lower than the value for wild-type pjDHFR, but the differences were not statistically significant (Table 3).

With TMP, the K37S (K_i , 81.6 nM) and K37S/F69S (K_i , 104.5 nM) substitutions that convert the pcDHFR enzyme to a more pjDHFR-like active site lowered the pcDHFR K_i from 305 nM toward the pjDHFR value (35 nM) (Table 3). Substitutions that convert hDHFR to have a more-pjDHFR-like active site lowered the hDHFR K_i from 4,093 nM toward the pjDHFR value for the

Q35S and N64S variants (1,350 and 738 nM, respectively) and the Q35S/N64S variant (K_i , 429 nM), but all three remained higher than the pjDHFR value (35 nM) (Table 3). Variants with substitutions converting human active-site residues to pcDHFR residues (Q35K and N64F) showed K_i values for TMP that were similar to each other (286 and 375 nM, respectively) and similar to the value for pcDHFR (K_i , 305 nM). The Q35K/N64F double variant did not show synergy (K_i , 585 nM), which differs significantly from the result for OAAG324.

Structure and ligand binding conformation of OAAG324. Overall, the crystal structures of the native and variant hDHFR enzymes resemble those previously reported and preserve the hydrogen bonding network, involving structural water, the conserved residues Thr136, Trp24, and Glu30, the N1 nitrogen, and the 2-amino group of OAAG324 (13, 14). Similarly, the 4-amino group of OAAG324 maintains contact with the conserved Ile7 and Tyr121 and with NADPH (see Fig. S1 in the supplemental material). The pyrido[2,3-*d*]pyrimidine ring orientation of OAAG324 is similar to that observed for other pyridopyrimidines (14, 27).

Inspection of the difference electron density map for the structure of the wild-type hDHFR binary complex shows OAAG324 bound in two alternate conformations of the 2N,5N-dichlorophe-

TABLE 3 Kinetic constants for selected antifolates against wild-type and variant pjDHFR, pcDHFR, and hDHFR

Variant	Enzyme	Mean $K_i \pm$ SEM (nM) of ^a :	
		TMP	OAAG324
Wild type			
0a	pjDHFR	35 $\pm$ 9 (6)	2.7 $\pm$ 0.4 (11)
0b	pcDHFR	305 $\pm$ 29* (15)	6.3 $\pm$ 2.0 (11)
0c	hDHFR	4,093 $\pm$ 1,000* (7)	24.4 $\pm$ 4 (12)
Mutant pjDHFR $\rightarrow$ pcDHFR			
1	S37K	Not available	Not available
2	S69F	82.2 $\pm$ 19.4* (4)	4.42 $\pm$ 0.17 (3)
3	S37K/S69F	28.0 $\pm$ 3.2 (9)	3.7 $\pm$ 0.9 (6)
Mutant pjDHFR $\rightarrow$ hDHFR			
4	S37Q	41.6 $\pm$ 4.8 (5)	1.46 $\pm$ 0.18 (5)
5	S69N	73.8 $\pm$ 8.1 (3)	5.45 $\pm$ 0.71 (3)
6	S37Q/S69N	27.6 $\pm$ 2.4 (4)	1.01 $\pm$ 0.09 (4)
Mutant pcDHFR $\rightarrow$ pjDHFR			
7	K37S	81.6 $\pm$ 10† (6)	0.8 $\pm$ 0.1† (3)
8	F69S	429 $\pm$ 64 (8)	8.31 $\pm$ 0.6 (9)
9	K37S/F69S	104.5 $\pm$ 8 (5)	1.42 $\pm$ 0.12† (6)
Mutant pcDHFR $\rightarrow$ hDHFR			
10	K37Q	32.6 $\pm$ 2.2† (7)	0.26 $\pm$ 0.03† (11)
11	F69N	Not available	Not available
12	K37Q/F69N	118 $\pm$ 14 (6)	0.71 $\pm$ 0.05† (13)
Mutant hDHFR $\rightarrow$ pjDHFR			
13	Q35S	1350 $\pm$ 170¥ (3)	8.4 $\pm$ 0.7 (3)
14	N64S	738 $\pm$ 140¥ (3)	11.4 $\pm$ 2 (3)
15	Q35S/N64S	429 $\pm$ 43¥ (4)	2.47 $\pm$ 0.5¥ (3)
Mutant hDHFR $\rightarrow$ pcDHFR			
16	Q35K	286 $\pm$ 33¥ (4)	16.7 $\pm$ 2 (4)
17	N64F	375 $\pm$ 64¥ (8)	0.58 $\pm$ 0.04¥ (6)
18	Q35K/N64F	585 $\pm$ 104¥ (8)	3.33 $\pm$ 0.12¥ (4)
Cross pjDHFR $\rightarrow$ pcDHFR/hDHFR			
19	S37K/S69N	25.4 $\pm$ 9.0 (5)	4.68 $\pm$ 0.48 (5)
20	S37Q/S69F	18.2 $\pm$ 2.1 (3)	1.13 $\pm$ 0.27 (3)
Cross pcDHFR $\rightarrow$ pjDHFR/hDHFR			
21	K37Q/F69S	123 $\pm$ 10 (6)	1.04 $\pm$ 0.06† (6)
22	K37S/F69N	227 $\pm$ 32 (9)	5.1 $\pm$ 0.38 (4)
Cross hDHFR $\rightarrow$ pjDHFR/pcDHFR			
23	Q35K/N64S	593 $\pm$ 44¥ (5)	9.63 $\pm$ 0.59 (3)
24	Q35S/N64F	617 $\pm$ 64¥ (10)	1.6 $\pm$ 0.06¥ (3)

^a The number of replicates in the determination is shown in parentheses. For statistical analysis, the values for each variant DHFR were compared only to the parental enzyme, e.g., variants of pjDHFR are compared to wild-type pjDHFR, variants of pcDHFR are compared to wild-type pcDHFR, and variants of hDHFR are compared to wild-type hDHFR. *, Statistically different from pjDHFR; †, statistically different from pcDHFR; ¥, statistically different from hDHFR.

nyl moiety, each refined at 50% occupancy (see Fig. S1 in the supplemental material). The absence of the cofactor NADPH in this structure opens the active-site volume and permits the alternative conformers of OAAG324 to bind (Fig. 3a). The two conformers differ significantly in the position of the 2*N*,5*N*-dichlorobenzyl ring. In conformer A (green molecule, Fig. 4a), the torsion angles about the methyl-amino bridge (see Table S3a) show that the pyrido[2,3-*d*]pyrimidine ring and the 2*N*,5*N*-dichlorophenyl

rings of the molecule are transoid to one another, with the 2'-chloro substituent toward the 4-amino side of the pyrido[2,3-*d*]pyrimidine ring, while in conformer B (cyan molecule, Fig. 4a), the two ring systems are cisoid, with the 2'-chloro substituent away from the 4-amino group (see Table S3a).

The structure of the Q35K single variant of hDHFR in ternary complex with NADPH shows that the conformation of OAAG324 (violet molecule, Fig. 3a) is similar to that of the cisoid conformer B observed in the native structure, although the rotation of the dichlorobenzyl ring places the dichloro substituents in different conformational spaces (Fig. 3a). The 2'-chlorine atom of the ternary complex in the Q35K single variant of hDHFR makes an intermolecular contact with one water molecule (3.6 Å), while the 5'-chlorine atom is in contact with two water molecules and the carbonyl backbone oxygen of N64 (2.9, 3.2, and 3.8 Å, respectively). The 2'-chlorine atom of conformer A of OAAG324 in hDHFR makes contact with a single water molecule (3.6 Å) and the carbonyl of S59 (3.6 Å), while the 5'-chlorine atom of conformer A makes a weak contact with the side chain N of N64 (4.6 Å) and to a water molecule (3.5 Å). The 2'-chlorine atom of conformer B makes only a single close contact with water (2.3 Å), and the 5'-chlorine atom makes a close contact to the side chain N of N64 (2.6 Å). The conformation of F31 in the wild-type hDHFR structure is such that it makes similar contacts to the 5'-chlorine atom (3.6 Å) in both conformers of OAAG324, whereas in the Q35K mutant hDHFR ternary structure, F31 has a different conformation and makes a weaker contact with the 5-chlorine atom of OAAG324 (4.3 Å) (Fig. 3a).

The structure of the Q35S/N64F double variant of hDHFR reveals a binary complex in which the conformation of OAAG324 is similar to that observed for conformer A (Fig. 3a, violet) of the wild-type binary complex. The major difference in the conformations of these two structures is that the 2',5'-dichlorophenyl ring is flipped with respect to the placement of the 2'- and 5'-chlorine atoms. This comes about because of the differences in the torsion angles about the bridging group (see Table S3a in the supplemental material). Thus, these conformational differences provide a region-specific environment for the two chlorine atoms.

Structural data for the K37Q single variant of pcDHFR as a ternary complex with NADPH and OAAG324 reveal interpretable density for the ligand and cofactor. However, the poor refinement statistics indicate that the crystal may be disordered. Nevertheless, this model reveals interactions similar to those observed in the human DHFR complexes with OAAG324 (Fig. 3b). These data show that the K37Q residue makes a close intermolecular contact to the 5'-Cl of OAAG324 (Q37 N...Cl, 3.0 Å) (Fig. 4).

Homology model of pjDHFR binding with OAAG324. To compare the binding of OAAG324 in the active site of pjDHFR with that observed for hDHFR, a homology model of the pjDHFR enzyme was calculated based on the crystal structure of the pcDHFR ternary complex with methotrexate and NADPH (28; M. Friendorf and T. Furlani, unpublished data). As illustrated in Figure 4, the conformation of OAAG324 from the homology modeling differs from that observed in the hDHFR complexes. The model of OAAG324 is displaced above the plane of the pyrido[2,3-*d*]pyrimidine ring observed in the hDHFR complexes, while the dichlorophenyl ring is oriented more like that observed for conformer A of the hDHFR binary complex (see Table S3a in the supplemental material). In addition, the conformation with

FIG 3 (a) Three structures are compared: OAAG324 (violet and yellow stick structures, conformers A and B, respectively) in the binary complex with hDHFR (violet ribbon structure, yellow amino acid side chains); OAAG324 (cyan stick structure) in the Q35K hDHFR single variant (cyan ribbons and amino acid side chains) NADPH ternary complex; and OAAG324 (green stick structure) in the Q35S/N64F double variant hDHFR (green ribbon and amino acid side chains) binary complex. The active-site residues in contact with OAAG324 are shown; they include Glu30, Phe31, Ser or Lys35, Arg70, Asn or Phe64, Ser59, and Leu22 (hDHFR numbering). The figure was drawn with PyMOL. (b) Comparison of three crystal structures: the OAAG324 (green and cyan stick structures) binary complex with wild-type hDHFR (green ribbon); OAAG324 (violet stick structure) in the ternary NADPH complex with the Q35K hDHFR variant (violet ribbon); and OAAG324 (yellow stick structure) in the ternary NADPH complex with the K37Q pcDHFR variant (yellow ribbon). The active-site residues Glu32, Lys/Gln37, and Asn/Phe69 (pcDHFR numbering) are highlighted; these are equivalent to Glu30, Lys/Gln35, and Asn/Phe64 in panel a. Drawn with PyMOL.

Met33 is similar to that of Phe31 as observed in the Q35K variant hDHFR ternary complex with OAAG324.

In the pjDHFR model with OAAG324 and NADPH, the closest contacts of the 5'-chlorine atom are to Ser69 (3.4 Å) and to Pro66 (3.7 Å), which is nearly the same as observed for the 5'-chlorine atom in the Q35S/N64F double variant to Pro61 (4.0 Å). In the double variant hDHFR complex, the contact of the 5'-chlorine atom to Phe64 is 4.3 Å, while in the pjDFHR model, the contact of the 5'-chlorine atom to Ser69 is 6.9 Å.

Structure and ligand binding conformation of TMP. The K37S/F69N double mutation of pcDHFR is a cross between the active site of pjDHFR (S37) and that of hDHFR (N64). The structural results for the TMP ternary complex with this double variant are similar to those observed for the native pcDHFR structure (Fig. 5) (18). As observed in many pcDHFR crystal structures (27), residues 1 to 4 are not observed in the electron density and the region between residues 83 to 89 is disordered.

The conformation of TMP is defined by two torsion angles about the methylene bridge, θ_1 and θ_2 [(C4-C5-C7-C1')]/(C5-C7-

FIG 4 Stereo comparison of the homology model of the pjDHFR ternary complex with NADPH and OAAG324 (green ribbons and sticks) with two crystal structures: hDHFR Q35K-NADPH with OAAG324 (pink ribbon and stick structure) and pcDHFR K37Q-NADPH with OAAG324 (yellow ribbon and stick structure). Active-site residues are labeled and include Asp32 (pjDHFR numbering, equivalent to Glu30 in hDHFR) and Glu32 (pcDHFR numbering, equivalent to Glu30 in hDHFR); Ser37 (pjDHFR numbering, equivalent to Gln35 in hDHFR), K37Q (pcDHFR numbering, equivalent to position 35 in hDHFR), and Q35K (hDHFR); and Ser69 (pjDHFR numbering, equivalent to Asn64 in hDHFR) and Phe69 (pcDHFR numbering, equivalent to Asn64 in hDHFR). Drawn with PyMOL.

C1'-C2') (Fig. 1), as well as the trimethoxybenzyl groups adopting an in-plane or out-of-plane conformation. Analysis of the TMP conformations observed in DHFR structures shows that they cluster in two broad torsion angle ranges, 180 to 210°/50 to 90° and 260 to 280°/95 to 105° for θ_1 and θ_2 , respectively (14, 17, 27). The cluster near 260 to 280°/95 to 105° is less populated, with only a few outliers with θ_2 near 50 to 65° for the two bridge angles. The bridging angles for the two molecules of TMP observed in the binary complexes with the Q35S/N64F and the Q35K/N64F double variants of hDHFR differ significantly from each other and have torsion angles near the extremes of the cluster at 270° and either 50° or 120° (see Table S3b in the supplemental material) (17). These conformations also differ from those reported for the structure of the pcDHFR ternary complex with TMP and NADPH (18) and the binary TMP complex with native hDHFR (16).

DISCUSSION

Positions 37 (or 35) and 69 (or 64) in the *P. jirovecii*, *P. carinii*, and human DHFR enzymes were confirmed in these studies to be significant targets for manipulation of the properties of these three forms of DHFR. Substitutions at these sites influenced both the

FIG 5 Comparison of TMP binding in pcDHFR (cyan ribbons and sticks) and the K37S/F69N double variant (green ribbons and sticks). Also highlighted are the active-site residues Glu32, Ile33, Lys/Ser37, Phe/Asn69, and Arg75 (pcDHFR numbering). Drawn with PyMOL.

intrinsic kinetic properties of the enzymes (Table 2) and the relative sensitivities of the enzymes to the competitive inhibitors TMP and OAAG324 (Table 3).

This study is the first report of kinetic data for the binding of OAAG324 (Fig. 1) to pjDHFR, pcDHFR, and hDHFR and its active-site variants; the kinetic data reported herein for the native enzymes and for TMP agree well with prior results published by us and others (7, 13). The results confirm that OAAG324 is a selective pjDHFR inhibitor, as originally suggested on the basis of IC_{50} data (15). The K_i values for OAAG324 were determined for all native and mutant DHFR forms from pj-, pc-, and hDHFR. Substitution of amino acids from the pjDHFR sequence into the active site of hDHFR lowered the K_i , i.e., improved the binding of OAAG324 (and thus, resulted in gain of function [29]) (Table 3 and Fig. 6a); the Q35S/N64S double variant shows a K_i value of 2.47 ± 0.5 nM (mean $\pm$ standard deviation), which is statistically significantly lower than the value for hDHFR (24.3 ± 4.4 nM) and is similar to that for pjDHFR (2.7 ± 0.4 nM). A similar pattern was seen for TMP, with the K_i values for both the N64S and Q35S/N64S variants being statistically significantly lower than the value for hDHFR (Table 3 and Fig. 6b). However, the K_i value for TMP, although reduced with respect to the value for hDHFR for the Q35S/N64S variant, remained more than 10-fold higher than the value for pjDHFR (Table 3), suggesting that these two residues may contribute more to the binding of OAAG324 to hDHFR than they do to the binding of TMP. This conclusion is also supported by the lower K_i values for the cross mutants that have one substitution from pcDHFR and the other from pjDHFR. The K_i value for the Q35S/N64F cross double variant is lower than that of pjDHFR itself (1.60 versus 2.7 nM). There is no discernible pattern in the binding of TMP to these cross variants, as their K_i values are similar to each other and intermediate between the values for hDHFR and pcDHFR (Table 3). The observation that the binding of OAAG324 is more selective with some of the hDHFR active-site variants than for native pjDHFR *in vitro* indicates that these variant models can provide useful insight into the role of these residues in controlling selectivity.

Analysis of the K_i values of OAAG324 for variants of hDHFR containing active-site residues from pcDHFR sequence again showed lowered K_i , i.e., improved binding of OAAG324 (Table 3 and Fig. 6a); in this case, the N64F single variant showed a K_i value of 0.58 ± 0.04 nM, which is significantly lower than the value for hDHFR (K_i , 24.4 nM) or pjDHFR (K_i , 2.7 nM). This pattern was not observed for TMP binding, for which analysis showed that both the Q35K and N64F single variants had similar values (K_i , 286 and 379 nM, respectively), which were 10-fold lower than that of hDHFR (K_i , $2,940 \pm 960$ nM). The K_i for TMP with the hDHFR Q35K/N64F double variant showed a higher value (604 ± 170 nM) than with the single variants.

The largest gain of sensitivity to OAAG324 was observed for the variants of pcDHFR containing active-site residues from hDHFR. In the case of K37Q pcDHFR, the K_i (0.26 nM) is significantly lower than the values for pcDHFR (6.3 nM), hDHFR (24.4 nM), or even pjDHFR (2.7 nM) (Table 3 and Fig. 6a). The structural data for the K37Q variant of pcDHFR in ternary complex with OAAG324 are consistent with these observations, as the Gln37 makes a close contact (N...5'-Cl, 3.0 Å) that can enhance binding. This interaction is not present in the pjDHFR structure that has a Ser at this position, nor for the wild-type pcDHFR in which Lys37 points away from the active-site pocket. Structural

FIG 6 (a) Plot of K_m of dihydrofolic acid (DHFA) versus K_i for OAAG324. This figure graphically summarizes the results showing that substituting amino acids from pcDHFR or pjDHFR active-site positions 35 and 64 into hDHFR primarily caused the human enzyme to become more sensitive to OAAG324. Substitutions into pcDHFR at these positions raised the K_m for DHFA and tended to lower the K_i for OAAG324. Changes induced by substitutions into pjDHFR were less dramatic than for the other two enzymes. (b) Plot of K_m of dihydrofolic acid (DHFA) versus K_i for TMP. Substituting amino acids from pcDHFR or pjDHFR active-site positions 35 and 64 into hDHFR primarily caused the human enzyme to become more sensitive to TMP. Substitutions into pcDHFR at these positions raised the K_m for DHFA and had various effects on K_i for TMP. Changes induced by substitutions into pjDHFR were less dramatic than for the other two enzymes.

models of the pjDHFR ternary complex with OAAG324 show weaker interactions of the 5'-Cl of OAAG324 with Ser69. Similar observations were made that helped explain the gain of function observed for the S108T mutation in *Plasmodium falciparum* DHFR (29).

These data for the binding of OAAG324 suggest that glutamine or lysine residues are virtually interchangeable in the active site with respect to inhibition by OAAG324: the K_i value of 16.7 ± 0.7 nM for OAAG324 with the Q35K variant is not significantly different from the K_i value of 24.3 ± 4.4 nM for hDHFR (Table 3, Fig. 6b). In contrast, the K_i value of 286 ± 33 nM for TMP with the Q35K variant is significantly lower than the K_i value of $2,940 \pm$

960 nM with hDHFR and, in fact, is very similar to the value with pcDHFR (305 nM). We conclude that although both TMP and OAAG324 show competitive inhibition patterns, the details of their catalytic mechanism of inhibition of DHFR are likely to differ.

The structural data for the binding of OAAG324 to the native hDHFR enzyme and its Q35K variant are consistent with their kinetic data. The intermolecular interactions of the chlorine atoms of the single conformer of OAAG324 bound to Q35K hDHFR NADPH ternary complex are tighter than those in the native hDHFR binary complex, as the loss of NADPH binding permits conformational flexibility in the binding of OAAG324. The alternate positions observed in the placement of the dichlorobenzyl ring of OAAG324 make weaker interactions and, in some instances, less favorable contacts with the surrounding residues. These data would predict a lower K_i value for OAAG324 with the Q35K variant than with hDHFR; the data show a slight (16.7 ± 6.7 versus 24.3 ± 4.4 nM, respectively) but not statistically significant decrease in potency.

In summary, these kinetic and structural data confirm that OAAG324 is a potent and selective inhibitor of pJDHFR *in vitro*. The potency of OAAG324 toward pJDHFR is 13-fold greater than the potency of TMP, but TMP is more selective (K_i hDHFR/ K_i pJDHFR is 117 for TMP and 9 for OAAG324). The strong interactive effects of variants at positions 35 and 64 of hDHFR suggest that these positions strongly influence both the potency and selectivity of the experimental compounds. In particular, the observation of nanomolar K_i values for OAAG324 with pJDHFR, as well as the subnanomolar binding to the K37Q pcDHFR variant, suggest that this compound is a good template for further optimization for the design of more selective and potent inhibitors of pJDHFR.

ACKNOWLEDGMENTS

Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL), a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences. We thank the beamline staff at SSRL and, also, Edward Snell for his help with data collection and processing. We also acknowledge Jennifer Makin, Jennifer Piraino, Jesse Nowak, and Elizabeth Stewart for their contributions to the mutagenesis and crystallization experiments in this project during their summer internships at HWI. We also acknowledge the excellent work of Pam Torkelson in conducting all enzyme assays reported in this study.

This work was supported in part by the following grants from the National Institutes of Health: grants GM051670 (V.C.), AI069966 (A.G.), and CA09885 (A.G.).

REFERENCES

- Kovacs JA, Gill VJ, Meshnick S, Masur H. 2001. New insights into transmission, diagnosis, and drug treatment of *Pneumocystis carinii* pneumonia. *JAMA* 286:2450–2460.
- Hughes WT. 1991. Prevention and treatment of *Pneumocystis carinii* pneumonia. *Annu. Rev. Med.* 42:287–295.
- Mu X-D, Wang G-F, Su L. 2011. A clinical comparative study of polymerase chain reaction assay for diagnosis of *Pneumocystis* pneumonia in non-AIDS patients. *Chin. Med. J.* 124:2683–2686.
- Reid AB, Chen SC, Worth LJ. 2011. *Pneumocystis jirovecii* pneumonia in non-HIV-infected patients: new risks and diagnostic tools. *Curr. Opin. Infect. Dis.* 24:534–544.
- Totet A, Duwat H, Magois E, Jounieaux V, Roux P, Raccourt C, Nevez G. 2004. Similar genotypes of *Pneumocystis jirovecii* in different forms of pneumocystis infection. *Microbiology* 150:1173–1178.
- Benfield T, Atzori C, Miller RF, Helweg-Larsen J. 2008. Second-line salvage treatment of AIDS-associated *Pneumocystis jirovecii* pneumonia. *J. Acquir. Immune Defic. Syndr.* 48:63–67.
- Ma L, Kovacs JA. 2000. Expression and characterization of recombinant human-derived *Pneumocystis carinii* dihydrofolate reductase. *Antimicrob. Agents Chemother.* 44:3092–3096.
- Nahimana A, Rabodonirina M, Bille J, Francioli P, Hauser PM. 2004. Mutations of *Pneumocystis jirovecii* dihydrofolate reductase associated with failure of prophylaxis. *Antimicrob. Agents Chemother.* 48:4301–4305.
- Chan DCM, Anderson AC. 2006. Towards species-specific antifolates. *Curr. Med. Chem.* 13:377–398.
- Gangjee A, Jain H, Kurup S. 2007. Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents, part I. *Anticancer Agents Med. Chem.* 7:523–542.
- Gangjee A, Jain H, Kurup S. 2008. Recent advances in classical and nonclassical antifolates as antitumor and antiopportunistic infection agents, part II. *Anticancer Agents Med. Chem.* 8:205–231.
- Gangjee A, Kurup S, Namjoshi O. 2007. Dihydrofolate reductase as a target for chemotherapy in parasites. *Curr. Pharm. Des.* 13:609–639.
- Cody V, Pace J, Makin J, Piraino J, Queener SF, Rosowsky A. 2009. Correlations of inhibitor kinetics for *Pneumocystis jirovecii* and human dihydrofolate reductase with structural data for human active site mutant enzyme complexes. *Biochemistry* 48:1702–1711.
- Cody V, Pace J, Chisum K, Rosowsky A. 2006. New insights into DHFR interactions: analysis of *Pneumocystis carinii* and mouse DHFR complexes with NADPH and two highly potent 5-(ω -carboxy(alkyloxy)) trimethoprim derivatives reveals conformational correlations with activity and novel parallel ring stacking interactions. *Proteins* 65:959–969.
- Gangjee A, Adair OO, Queener SF. 2003. Synthesis and biological evaluation of 2,4-diamino-6-(arylaminoethyl)pyrido[2,3-*d*]pyrimidines as inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* dihydrofolate reductase and as antiopportunistic infection and antitumor agents. *J. Med. Chem.* 46:5074–5082.
- Cody V. 2002. Structure analysis of human dihydrofolate reductase complexes with trimethoprim and epiroprim. *Acta Crystallogr. A* 58(Suppl): C288.
- Cody V, Pace J, Pirano J, Queener SF. 2011. Crystallographic analysis reveals a novel second binding site for trimethoprim in active site mutants of human dihydrofolate reductase. *J. Struct. Biol.* 176:52–59.
- Champness JN, Achari A, Ballantine SP, Bryant PK, Delves CJ, Stammers DK. 1994. The structure of *Pneumocystis carinii* dihydrofolate reductase to 1.9 Å resolution. *Structure* 2:915–924.
- McPhillips TM, McPhillips SE, Chiu HJ, Cohen AE, Deacon AM, Ellis PJ, Garman E, Gonzalez A, Sauter NK, Phizackerley RP, Soltis SM, Kuhn P. 2002. Blu-ice and the distributed control system: software for data acquisition and instrument control at macromolecular crystallography beamlines. *J. Synchrotron Radiat.* 9:401–406.
- Cohen AE, Ellis PJ, Miller MD, Deacon AM, Phizackerley RP. 2002. An automated system to mount cryo-cooled protein crystals on a synchrotron beamline using compact sample cassettes and a small-scale robot. *J. Appl. Crystallogr.* 35:720–726.
- Gonzalez A, Moorhead P, McPhillips SE, Song J, Sharp K, Taylor JR, Adams PD, Sauter NK, Soltis SM. 2008. Web-ice: integrated data collection and analysis for macromolecular crystallography. *J. Appl. Crystallogr.* 41:176–184.
- Cody V, Luft JR, Pangborn W. 2005. Understanding the role of leu22 variants in methotrexate resistance: comparison of wild-type and leu22arg variant mouse and human dihydrofolate reductase ternary crystal complexes with methotrexate and NADPH. *Acta Crystallogr. D* 61:147–155.
- Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AG, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS. 2011. Overview of the CCP4 suite and current developments. *Acta Crystallogr. D* 67:234–242.
- Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of COOT. *Acta Crystallogr. D* 66:486–501.
- Schuettelkopf AW, van Aalten DMF. 2004. PRODRG: a model tool for

- high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr. D* **60**:1355–1363.
26. Lovell SC, Davis IW, Arendall WB, III, de Bakker PI, Word JM, Prisant MG, Richardson JS, Richardson DC. 2003. Structure validation by α geometry: phi, psi and $\text{C}\beta$ deviation. *Proteins* **50**:437–450.
 27. Cody V, Schwalbe CH. 2006. Structural characteristics of antifolate dihydrofolate reductase enzyme interactions. *Crystallogr. Rev.* **12**:301–333.
 28. Cody V, Galitsky N, Rak D, Luft JR, Pangborn W, Queener SF. 1999. Ligand induced conformational changes in the crystal structures of *Pneumocystis carinii* dihydrofolate reductase complexes with folate and NADP^+ . *Biochemistry* **38**:4303–4312.
 29. Mharakurwa S, Kumwenda T, Mkulama MAP, Musapa M, Chishimba S, Shiff CJ, Sullivan DJ, Thuma PE, Liu K, Agre P. 2011. Malaria antifolate resistance with contrasting *Plasmodium falciparum* dihydrofolate reductase (DHFR) polymorphisms in humans and *Anopheles* mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* **108**:18796–18801.