

Towards New Antifolates Targeting Eukaryotic Opportunistic Infections[∇]

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Trimethoprim, an antifolate commonly prescribed in combination with sulfamethoxazole, potently inhibits several prokaryotic species of dihydrofolate reductase (DHFR). However, several eukaryotic pathogenic organisms are resistant to trimethoprim, preventing its effective use as a therapeutic for those infections. We have been building a program to reengineer trimethoprim to more potently and selectively inhibit eukaryotic species of DHFR as a viable strategy for new drug discovery targeting several opportunistic pathogens. We have developed a series of compounds that exhibit potent and selective inhibition of DHFR from the parasitic protozoa *Cryptosporidium* and *Toxoplasma* as well as the fungus *Candida glabrata*. A comparison of the structures of DHFR from the fungal species *Candida glabrata* and *Pneumocystis* suggests that the compounds may also potently inhibit *Pneumocystis* DHFR.

Reduced folate cofactors such as tetrahydrofolate are required for critical cellular functions, such as the production of dTMP, several amino acids, and purines. Dihydrofolate reductase (DHFR), the sole source of tetrahydrofolate, is one of several enzymes in the folate biosynthetic pathway. DHFR has been a recognized and validated drug target since the 1960s, with the discovery of methotrexate (5). Fortunately, since pathogenic and human forms of DHFR exhibit several critical sequence differences, it has also been possible to develop species-selective antifolates for several infectious diseases, including malaria, toxoplasmosis, and urinary tract infections. Trimethoprim (TMP) (Fig. 1) is a commonly administered antifolate, primarily in combination with sulfamethoxazole (TMP-SMZ), which inhibits dihydropteroate synthase (DHPS), another enzyme in the folate pathway (14). TMP-SMZ is most effective against prokaryotic pathogens. However, it is also recognized as first-line therapy in treating and preventing the common eukaryotic opportunistic pathogen *Pneumocystis jirovecii*, which causes life-threatening pneumonia in immunocompromised patients (20).

Interestingly, while TMP inhibits bacterial species of DHFR at concentrations in the low nanomolar range, it inhibits many eukaryotic species of the enzyme at concentrations in the micromolar range, resulting in 3 orders of magnitude lower potency. Even the use of TMP-SMZ as a prophylactic agent against pneumocystis relies heavily on the sulfa component (31) and only on relatively weak binding between TMP and *P. jirovecii* DHFR (6, 18). In fact, studies have reported that mutations conferring resistance to TMP-SMZ arise in DHPS, not in DHFR (17, 28). In contrast, when the DHFR inhibitor pyrimethamine, which is four times more potent than TMP (18), is used in combination with sulfadiazine against pneumocystis, mutations arise in DHFR as well as DHPS (20). A logical conclusion of these studies is that the low potency of TMP against *P. jirovecii* DHFR is preventing it from reaching its full potential as an effective therapy for this eukaryotic

opportunistic pathogen. In contrast, high-affinity DHFR inhibitors such as trimetrexate and piritrexim potently inhibit the growth of cultures of *Pneumocystis* (2, 23) even when administered as single agents. These inhibitors have limited use, however, because of their toxicity to the human enzyme.

In an attempt to discover more potent analogs of TMP that would eliminate the need for the sulfonamide component and selectively inhibit DHFR from *Pneumocystis* and *Toxoplasma*, several DHFR inhibitors have been developed over the past 2 decades (10–12, 24–27). At the time, investigators were examining interactions with *Pneumocystis carinii* instead of *Pneumocystis jirovecii*, primarily because of the late recognition of *P. jirovecii* as the causative agent of human pneumocystis pneumonia during compound development and the ability to use the *P. carinii* DHFR crystal structures (7–9) in design. During this time, compounds were developed that exhibited nanomolar levels of inhibition for the *Toxoplasma gondii* and *P. carinii* enzymes.

We have been building a program to reengineer TMP to more potently inhibit eukaryotic species of DHFR as a viable strategy for new drug discovery targeting several eukaryotic opportunistic pathogens. These novel DHFR inhibitors, originally based on the TMP scaffold, exhibit potent inhibition of DHFR from the parasitic protozoa *Cryptosporidium* and *Toxoplasma* and the fungus *Candida glabrata*. We chose to study these opportunistic infections because for immunocompromised patients there are no approved agents for treatment of cryptosporidiosis (1, 30), the severity of toxoplasmosis can be devastating (3), and strains of *Candida* (such as *C. glabrata*) resistant to the commonly administered antifungals now comprise at least 20% of candidemia cases (13, 22, 29). Herein, we analyze the interactions of these inhibitors across a wide variety of eukaryotic opportunistic pathogens and extend this analysis to *Pneumocystis jirovecii* DHFR. A comparison of the structures of *Candida glabrata* and *Pneumocystis carinii* DHFR, along with a sequence comparison of *P. jirovecii* and *P. carinii*, suggests that these compounds may additionally inhibit *Pneumocystis jirovecii* DHFR.

MATERIALS AND METHODS

***Cryptosporidium hominis* and *T. gondii* DHFR-TS preparation.** DHFR-thymidylate synthase (DHFR-TS) from *C. hominis* (ChDHFR-TS) was expressed in *Escherichia coli* and purified using a methotrexate agarose column (Sigma).

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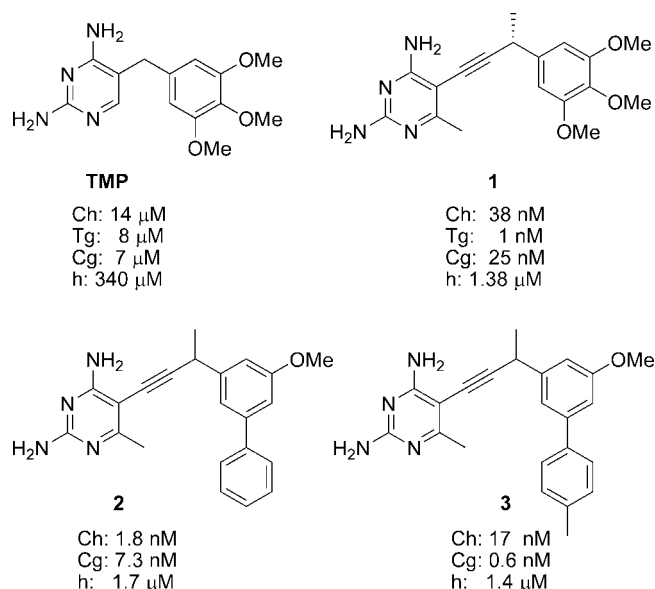


Fig. 1. Antifolates and their corresponding IC_{50} s against various eukaryotic species of DHFR. Ch, *Cryptosporidium hominis*; Tg, *Toxoplasma gondii*; Cg, *Candida glabrata*; h, human.

Briefly, crude cell lysate was loaded on the column, which was then washed with 4 column volumes of buffer A [0.2 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.0, 1 mM EDTA, 5 mM dithiothreitol] containing 0.2 M NaCl, followed by 8 column volumes of buffer A containing 0.5 M NaCl. The protein was eluted from the column with buffer A containing 2 mM dihydrofolate. The gene for DHFR from *T. gondii* was cloned and the protein expressed and purified as previously described (21).

***C. glabrata* DHFR preparation, crystallization, and structure solution.** The gene for *C. glabrata* DHFR (CgDHFR) was amplified from genomic DNA obtained from ATCC (36909D), inserted into a pET41 vector with a C-terminal His tag, and expressed in *E. coli* BL21(DE3) cells. The protein was purified using nickel affinity chromatography, desalted with a PD-10 column, and concentrated to 13 mg/ml. CgDHFR was incubated with 1.5 mM NADPH and 1 mM inhibitor and crystallized by hanging drop vapor diffusion using a mother liquor with 0.1 M Tris, pH 8.5, 30% polyethylene glycol 4000, and 0.2 M $MgCl_2$. Diffraction data were collected at Brookhaven National Laboratory, beam line X25, and processed using the program HKL2000. The structure was determined by molecular replacement, using a structure of *C. albicans* DHFR (32), and refined using the program Refmac5 (19). Synthesis and characterization of compounds 1 to 3 and their analogs have been described previously (16, 21).

Enzyme assays. Enzyme assays were performed at 25°C by monitoring the rate of NADPH oxidation at 340 nm over 5 min. Reactions were performed in the presence of 20 mM TES, pH 7.0, 50 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 100 μ M NADPH, and 1 mM dihydrofolate.

Protein structure accession number. The coordinates for the CgDHFR protein have been deposited at the Protein Data Bank with identifier 3CSE.

RESULTS AND DISCUSSION

Unlike prokaryotic, fungal, and human species of DHFR, parasitic protozoa, including *C. hominis* and *T. gondii*, have a bifunctional form of the enzyme (DHFR-TS), wherein DHFR and TS are translated from the same polypeptide. The DHFR and TS domains are distinct, and the active sites are separated by approximately 46 Å, allowing inspection of each active site individually. Therefore, in order to design more effective DHFR inhibitors for eukaryotic pathogens, we first examined the crystal structure of ChDHFR (4), with TMP modeled in the active site. It was evident from this analysis that the trime-

thoxyphenyl ring of TMP did not form maximal contacts with the hydrophobic pocket that normally houses the *para*-aminobenzoic acid moiety of the substrate, dihydrofolate (residues Thr 58, Ser 61, Ile 62, and Leu 67). Specifically, there appeared to be at least a 1- to 2-Å gap between the trimethoxyphenyl ring and the ideal distance for van der Waals contacts with residues Ile 62, Thr 58, and Ser 61, thereby reducing the potential van der Waals energy. We hypothesized that new inhibitors with an extended bridge between the diaminopyrimidine and the trimethoxyphenyl rings of TMP may increase the favorable contacts between the phenyl ring and the residues in the hydrophobic pocket. After synthesizing a series of analogs to probe the proper conformational freedom and length of the extended compounds, we found that the propargyl-linked compound 1 (Fig. 1) exhibited 368-fold greater potency than that of TMP against the ChDHFR enzyme (21). Compound 1 then formed the basis of new lead discovery for potent antifolates effective against eukaryotic pathogens.

We then constructed homology models of DHFR from *Toxoplasma gondii* and *Candida glabrata* (TgDHFR and CgDHFR) and predicted that the extended TMP compounds may be effective inhibitors of these eukaryotic DHFR enzymes as well. Excitingly, *in vitro* enzyme inhibition assays with TgDHFR, CgDHFR, and compound 1 confirmed that this scaffold could serve as a base structure of an inhibitor for these species of DHFR, since 50% inhibitory concentrations (IC_{50} s) were 1 and 25 nM, respectively (Fig. 1) (16, 21).

We crystallized the *C. glabrata* DHFR enzyme with an analog of compound 1 (a compound with a dimethoxyphenyl ring and an ethyl group at the C-6 position of the pyrimidine instead of a methyl) in order to validate the proposed binding mode and to gain new insights for future inhibitor design. The structure (PDB accession number 3CSE) was determined to 1.6-Å resolution and was refined to an *R* factor of 0.18 and an R_{free} of 0.23. The structure directly confirms that the inhibitor binds in the active site, as predicted (Fig. 2) (16). With the extended bridge between the two rings, the dimethoxyphenyl ring reaches deeper into the eukaryotic pocket than a single methylene bridge and interacts with several eukaryotic hydrophobic residues, including Thr 58, Ser 61, Ile 62, and Pro 63 of CgDHFR. There appear to be ideal distances between the van der Waals radii of the residues and the inhibitor, explaining the large increase in potency.

Using this first crystal structure of CgDHFR bound to one of the novel antifolates and interpreting docked complexes of compound 1 bound to ChDHFR, we designed second-generation inhibitors that have increased potency for the pathogenic enzymes. Compound 2 has an IC_{50} of 1.8 nM against ChDHFR (a 21-fold improvement relative to compound 1), and compound 3 exhibits picomolar inhibition against CgDHFR (a 42-fold improvement relative to compound 1). These new compounds also exhibit potent inhibition of the growth of *C. glabrata* in culture, with a MIC of 1.5 μ g/ml (16).

Subtle differences in eukaryotic species of DHFR also allow for selectivity against the human form of the enzyme. In fact, compounds 1 to 3 are poor inhibitors of the human enzyme, with IC_{50} s in the micromolar range (Fig. 1). There are several residue substitutions between the opportunistic pathogens and human DHFR (hDHFR), two of which are critical in the active site. Near the pyrimidine ring, CgDHFR has Met 33 and

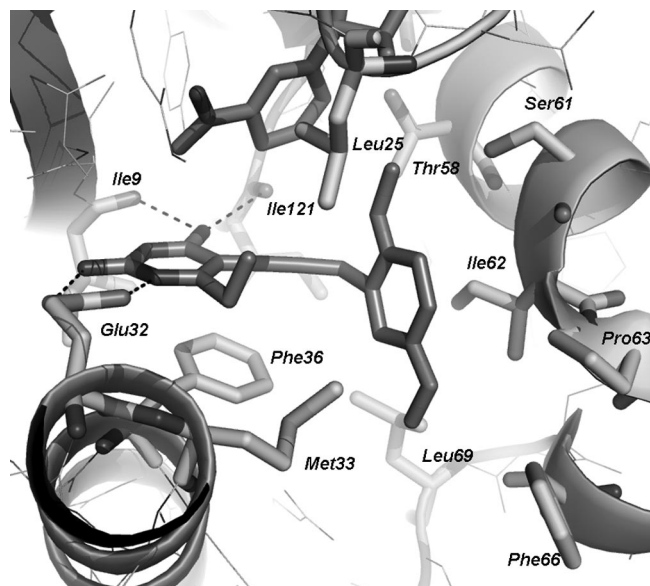


FIG. 2. The crystal structure of CgDHFR bound to an analog of compound 1 and NADPH reveals details of the interaction between the ligand and the eukaryotic hydrophobic pocket. Hydrogen bonds are shown with dashed lines.

ChDHFR has Leu 33, while hDHFR has the much larger and less flexible residue Phe 31. Near the opening to the active site, there is a loop in hDHFR, containing residues Pro 61 to Asn 64, which is absent in ChDHFR. While the loop is present in CgDHFR (Pro 63 to Phe 66), it is translated by 1.2 Å and contains a hydrophobic Phe residue instead of the polar Asn residue in hDHFR. The structural differences translate into potency differences for inhibition of the enzyme (Fig. 1) and growth of the cell culture. Compound 3 inhibits the growth of *Candida glabrata* 13.5-fold more potently than the growth of human cell lines (16). Cellular selectivity indices of >10 are considered to be within an acceptable range (15), although obviously further designs will focus on increasing this selectivity.

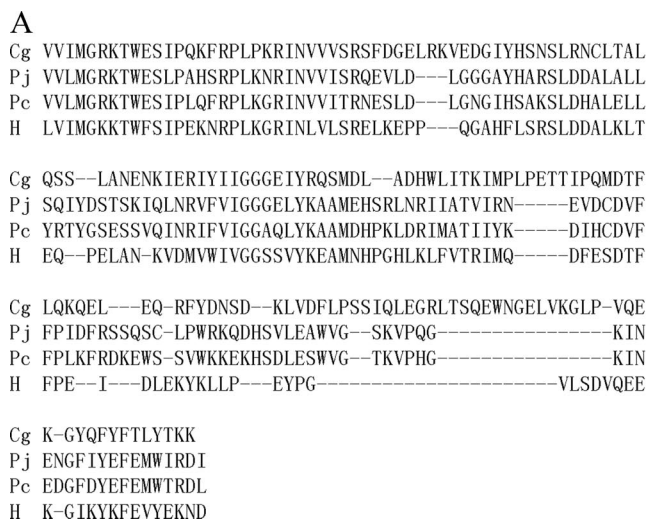
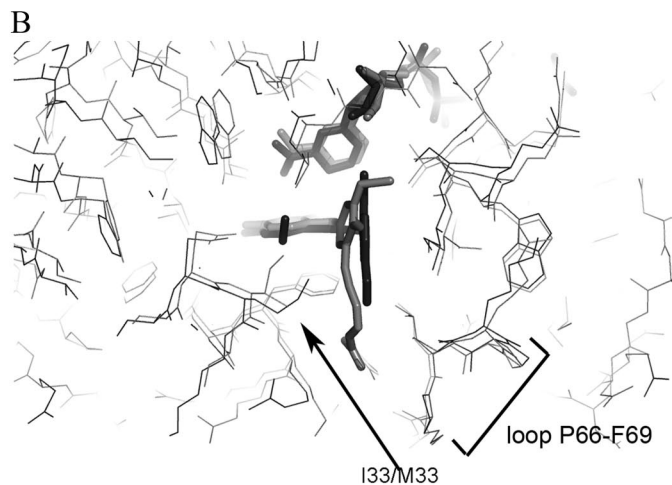


FIG. 3. Comparison of sequences of DHFR from *C. glabrata* (CgDHFR), *P. jirovecii* (PjDHFR), *P. carinii* (PcDHFR), and human (hDHFR) (A) and of the structures of CgDHFR (black) and PcDHFR (gray) bound to antifolates and NADPH (ligands are shown in stick form) (B).

Interestingly, in comparing compounds 1 and 3, the improvement in potency (42-fold) at the enzyme level is in the same range as the improvement in potency of growth inhibition (13-fold). This correlation suggests that high enzyme potency is an important factor in developing an efficacious drug lead. In addition to correlations between potency at the level of the enzyme and fungal growth inhibition, there appear to be correlations between the MIC and the hydrophobic character of the compounds, suggesting that we can optimize cell permeation in future generations of compound design.

As discussed in the introduction, TMP-SMZ is used prophylactically for *Pneumocystis pneumonia*, despite the weak affinity of TMP for *Pneumocystis* DHFR ($IC_{50} = 4.8 \mu\text{M}$ for *P. jirovecii* DHFR) (18). Since the propargyl-based antifolates are significantly more potent than TMP against the fungal *C. glabrata* DHFR, they may also be effective against the fungal *Pneumocystis* DHFR. In order to begin to explore whether these compounds may exhibit in vitro potency for *Pneumocystis* DHFR, we compared the sequences of *P. carinii* DHFR (PcDHFR), *P. jirovecii* DHFR, and *C. glabrata* DHFR (Fig. 3A). We also compared the available structures (Fig. 3B) of *P. carinii* DHFR (9) and *C. glabrata* DHFR (16).

Overall, the structures of the fungal *C. glabrata* and *P. carinii* enzymes are very similar and superimpose with a root mean square deviation of 3.6 Å over 161 C- α atoms. The active site residues exhibit even more structural similarity (Fig. 3B), reinforcing the similarity between these enzymes. The only residue difference between CgDHFR and PcDHFR is located at Met 33 (CgDHFR) and Ile 33 (PcDHFR). Interestingly, *P. jirovecii* DHFR maintains the methionine at position 33 (Fig. 3A). Specifically, it is likely that the propargyl-linked antifolates will be effective inhibitors of *Pneumocystis* DHFR based on the similarity of the active site size and the apparent fit of the docked lead to the *Pneumocystis* enzyme. The compounds are likely to maintain their specificity for the pathogenic species of DHFR over hDHFR, since the key loop at the active site, residues Pro 66 to Phe 69 in PcDHFR, maintains the same position and conformation as those observed in CgDHFR and



includes the Phe residue, as opposed to the Asn residue in hDHFR. When the structure of *P. jirovecii* DHFR becomes available, it will be important to include it in a wider comparison with other fungal enzymes.

In conclusion, DHFR is a potentially effective drug target for opportunistic infectious diseases. We exploited the overall similarities in the structures of eukaryotic species of DHFR to design a series of antifolates that include an extended propargyl linker, intended to force the interaction of the substituted phenyl ring with the eukaryotic hydrophobic pocket. These novel antifolates are between 300- and 11,000-fold more potent than TMP against the eukaryotic pathogenic enzymes. A high-resolution crystal structure of CgDHFR bound to one of these extended compounds confirms that the inhibitor is bound to the active site, with the phenyl ring buried in a hydrophobic pocket, accounting for the increased affinity. Using the subtle differences between eukaryotic species of DHFR, we customized the lead compounds and synthesized very potent and selective second-generation inhibitors for ChDHFR and CgDHFR. A structural comparison with *Pneumocystis* DHFR suggests that the propargyl-linked antifolates may also be effective inhibitors of this important pathogen.

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