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X-ray structure of the ternary MTX•NADPH complex of the anthrax dihydrofolate reductase: a pharmacophore for dual-site inhibitor design

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Abstract

For reasons of bioterrorism and drug resistance, it is imperative to identify and develop new molecular points of intervention against anthrax. Dihydrofolate reductase (DHFR) is a highly conserved enzyme and an established target in a number of species for a variety of chemotherapeutic programs. Recently, the crystal structure of *B. anthracis* DHFR (baDHFR) in complex with methotrexate (MTX) was determined and, based on the structure, proposals were made for drug design strategies directed against the substrate binding site. However, little is gleaned about the binding site for NADPH, the cofactor responsible for hydride transfer in the catalytic mechanism. In the present study, X-ray crystallography at 100 K was used to determine the structure of baDHFR in complex with MTX and NADPH. Although the NADPH binding mode is nearly identical to that seen in other DHFR ternary complex structures, the adenine moiety adopts an off-plane tilt of nearly 90° and this orientation is stabilized by hydrogen bonds to functionally conserved Arg residues. A comparison of the binding site, focusing on this region, between baDHFR and the human enzyme is discussed, with an aim at designing species-selective therapeutics. Indeed, the ternary model, refined to 2.3 Å resolution, provides an accurate template for testing the feasibility of identifying dual-site inhibitors, compounds that target both the substrate and cofactor binding site. With the ternary model in hand, using *in silico* methods, several compounds were identified which could potentially form key bonding contacts in the substrate and cofactor binding sites. Ultimately, two structurally distinct compounds were verified that inhibit baDHFR at low μM concentrations. The apparent K_d for one of these, (2-(3-(2-(hydroxyimino)-2-(pyridine-4-yl)-6,7-dimethylquinoxalin-2-yl)-1-(pyridine-4-yl)ethanone oxime), was measured by fluorescence spectroscopy to be 5.3 μM.

Keywords

Anthrax; X-ray crystallography; nucleotide biosynthesis; dual-site inhibition; antifolate; structure-based drug design; cryocrystallography

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Coordinates for the baDHFR•MTX•NADPH structure have been deposited in the Protein Data Bank under the accession number 3DAT. The authors declare that they have no competing financial interests.

1. Introduction

Anthrax has emerged as an agent of bioterrorism that has to be dealt with immediately due to the current threat levels. The search for new therapeutic agents against anthrax is important because current vaccination strategies require regular administration of boosters over an 18 month period for maintaining immunity (Spencer, 2003; Swartz, 2001), and prophylactic treatment of anthrax necessitates that the patients be administered with antibiotics prior to the manifestation of symptoms (Coker et al., 2002). Another concern in the treatment of anthrax is the age restriction of the patients; ciprofloxacin is not licensed for use in children (Grady, 2005). Furthermore, some of these therapies have serious limitations due to resistance (Doganay and Aydin, 1991; Inglesby et al., 1999). *B. anthracis* is naturally resistant to trimethoprim (TMP) (Barrow et al., 2004), at least in part due to the presence of a Phe 96 substitution in the putative TMP binding site of dihydrofolate reductase (DHFR) (Bennett et al., 2007), a nearly universally conserved enzyme in the folate biosynthesis pathway (Kraut and Matthews, 1987). In most other prokaryotes, at this position exists either an Ile or Val; the Phe side chain in the *B. anthracis* DHFR (baDHFR) active site sterically clashes with the alkyl linker of TMP (Bennett et al., 2007), possibly attributing to the enzyme's inherent resistance. In addition to TMP, anthrax is also naturally resistant to sulfonamides and some cephalosporins. The standard prophylaxis after anthrax exposure is the second-generation fluoroquinolone ciprofloxacin (Grady, 2005), a topoisomerase inhibitor, which is not licensed for use in children. Further, the presence and continuous development of drug resistance requires for the scientific community to continue development of new drug antimicrobial agents (Friedlander, 2001).

DHFR is required for *de novo* DNA synthesis and amino acid synthesis and has been a well established anti-cancer and anti-fungal target for half a century (Huennekens, 1996) (Huennekens, 1994). This is because nearly all prokaryotes must synthesize folate compounds *de novo*, starting with GTP and utilizing several different enzymes in a multi-step pathway (Hawser et al., 2006; Hitchings, 1989). The general importance of DHFR is in its recycling of tetrahydrofolate (THF), a necessary cofactor used in several diverse biosynthetic pathways where one-carbon transfer reactions occur, such as in thymine and methionine synthesis (Huennekens, 1996). It does this by catalyzing the NADPH-dependent reduction of 7,8-dihydrofolate (DHF), producing 5,6,7,8-THF (Benkovic, 1980). The inhibition of DHFR depletes THF pools, causing genomic and proteomic instability and, ultimately, cell death (Huennekens, 1994). Similarly, baDHFR is a valid drug target because it is essential for the survival and pathogenesis of anthrax (Barrow et al., 2004; Joska and Anderson, 2006). Thus, baDHFR is an attractive antimicrobial drug target. Common DHFR inhibitors used in a clinical setting include TMP, methotrexate (MTX; one of the first chemotherapeutic agents discovered and is still used actively in the clinic against certain cancers and rheumatoid arthritis) and pyrimethamine (PYR; an antimalarial) (Hekmat-Nejad and Rathod, 1997; Huennekens, 1994; Katzung and Trevor, 2004). It should be noted that all of these target the substrate binding site. Very recent efforts by multiple laboratories to inhibit baDHFR have also focused on exploiting this site: structurally diverse sets of compounds were generated, many of which show efficacy *in vitro* (with IC₅₀ values in the nM range) and a few which prove effective *in vivo* (with MICs in the µg/ml range) (Barrow et al., 2007; Joska and Anderson, 2006). However, to the best of the authors' knowledge, until recently, there have been no drugs developed so far against DHFR with the aim of dual-site inhibition. These, of course, would be compounds that can at least partially overlap the substrate and cofactor binding sites of DHFR; these are bi-functional in a sense that they can bind two distinct sites on the one enzyme molecule. (Thus, we are not considering here inhibitors which can bind two different targets in the same pathway, which can be termed dual-target inhibitors, such as those developed against the DHFR-thymidylate synthase bi-functional enzyme from *Plasmodium* species (Yuthavong et al., 2005; Yuvaniyama et al., 2003)). To avoid confusion, we adopt the term "dual-site" inhibitors

here to mean compounds which can bind in and bridge both of the substrate and cofactor binding sites of DHFR). Indeed, a recent kinetic and structural study of *Mycobacterium tuberculosis* DHFR (mtDHFR) revealed that a particular isomer of an NADP-adduct of isoniazid, the well-known and classic anti-tuberculosis drug (Katzung and Trevor, 2004), binds to and potently inhibits mtDHFR (Argyrou et al., 2006). The isonicotinyl ring (the isoniazid-nicotinamide adduct) projects into the substrate binding site, packs amongst multiple hydrophobic residues and actually forms a second sphere hydrogen bond through a water molecule to the active site Asp 27 residue (Argyrou et al., 2006), confirming dual-site inhibition.

The knowledge-based approach has been accepted as a useful tool in drug design. Moderate resolution (at least 2.4 Å) three-dimensional X-ray structures of drug targets are a prerequisite for knowledge-based drug design (Blundell, 1996). These structures provide a starting point for rational drug design by defining the topography of the protein target that the ligand surface must complement. This information helps the synthetic chemist optimize compounds so as to enhance their interactions with the protein, resulting in improved potency and selectivity (Greer et al., 1994). Indeed, there are now several drugs on the market that originated from this structure-based design approach. The most commonly cited are the human immunodeficiency virus (HIV) drugs (Leonard, 1996), such as amprenavir (Agenerase) and nelfinavir (Viracept), which were developed using the crystal structure of HIV protease (Greer et al., 1994). Such an approach could be useful for developing baDHFR inhibitors.

Previously, we reported the structure of baDHFR bound to the inhibitor and anti-cancer agent MTX (Bennett et al., 2007), an antifolate which binds in the normal substrate binding site. In the present study we report the ternary structure of baDHFR bound to MTX•NADPH (Figs. 1, 2). As parts of the NADPH cofactor and the MTX or DHF ligand binding sites are proximal to each other, the development of dual-site inhibitors are an attractive prospective. In the past, dual-site inhibitors have been developed against several drug targets (Haviv et al., 2007; Munoz-Ruiz et al., 2005; Wolin et al., 2008). Dual-site inhibitors offer the possibility of improving potency through avidity effects. Additionally, one may evade resistance more effectively from the host if exploiting two distinct and disparate binding sites on the enzyme template. To further pursue this using the knowledge-based approach, we conducted an *in silico* virtual screen using the MTX•NADPH binding site as defined by our baDHFR ternary crystal structure. Through iterative virtual screening procedures, we were able to narrow down the number of hits to 212. These hits were docked into the baDHFR active site and, subsequently, 15 candidates were tested for baDHFR inhibition. Two compounds showed inhibition of baDHFR activity. These initial hits are being used as lead compounds for developing highly potent dual-site baDHFR inhibitors.

2. Materials and methods

2.1 Protein expression, purification and crystallization

Expression and purification of baDHFR as well as subsequent crystallization of the binary complex with MTX was performed as described previously (Bennett et al., 2007). Attempts to cocrystallize NADPH with baDHFR have proven unsuccessful. Therefore, we turned to soaking as a way to solve a ternary complex structure. To form the baDHFR ternary complex with MTX and NADPH, crystals of baDHFR:MTX were soaked at 4°C for 8 hours in harvesting buffer (0.1 M Bis-Tris (pH 5.5), 0.2 M CaCl₂ and 22.5% (w/v) PEG 3350) supplemented with 5 mM β-NADPH (Alexis Pharmaceuticals). At the completion of soaking, crystals were cryoprotected for 15–30 s in harvesting buffer with 20% (v/v) glycerol and 5 mM β-NADPH and, subsequently, were frozen for storage in liquid nitrogen (LN₂).

2.2 X-ray crystallography

Synchrotron X-ray ($\lambda = 0.9\text{\AA}$) diffraction data was collected at 100 K at beam line 14-ID-B of the BioCARS station at the Advanced Photon Source within Argonne National Laboratory (Argonne, IL). A full 360° ($0.5^\circ/\text{oscillation}$) of diffraction data was collected on a MAR CCD 165 detector; the visual diffraction resolution was beyond $2.0\text{\AA}$. Only the first 150° wedge of data was used for the final scaling (to $2.3\text{\AA}$ resolution) in HKL2000 (Otwinowski and Minor, 1997) and to generate the mtz file for refinement, due to significantly higher R_{symm} and χ^2 values for frames beyond this. The R_{merge} and completeness to $2.3\text{\AA}$ is 14.3% and 93.8%, respectively. The ternary baDHFR•MTX•NADPH structure was solved by the Difference Fourier method and the structure was refined using Refmac5 (Murshudov et al., 1997) (ver. 5.2.0019; implemented in CCP4 version 6.0 (CCP4, 1994)) using the baDHFR•MTX binary structure (PDB ID: 2QK8) as the starting coordinate set. Further refinement was performed using maximum likelihood restrained refinement in Refmac5 using TLS restraints. DHFR has been described as having a structure composed of two “subdomains” (Sawaya and Kraut, 1997), distinct regions for substrate-binding and for adenosine cofactor-binding. Thus, the structure was divided into three TLS rigid bodies: 1–36, 37–108 and 109–162. The first and last groups correspond to the substrate-binding subdomain whereas the middle group corresponds to the adenosine cofactor-binding subdomain. Refinement with these TLS restraints resulted in a 2–3% drop in R_{free} . Model building in Coot (Emsley and Cowtan, 2004), addition of solvent and refinement (xyz positional and isotropic B-factor) were interspersed for several cycles until the R_{free} eventually decreased to 27.3%.

2.3 Virtual screening

We used the Sybyl8.0 suite for all *in silico* screening using the Open NCI Database of small molecules (<http://cactus.nci.nih.gov/ncidb2>). Many of these compounds are available free to academic researchers through the Open Chemical Repository, managed by the Developmental Therapeutics Program (<http://dtp.nci.nih.gov>). For the Unity search using the Open NCI library, a $5\text{\AA}$ sphere radius around the MTX and NADPH ligands was used to define the baDHFR active site. Three hydrophobic surfaces were implemented in the Unity search (the nicotinamide ring of NADPH, the diaminopyrimidine ring of the pteridine moiety of MTX and the *p*-ABA ring of MTX), each with a constraint value of $1.0\text{\AA}$ (see Fig. 4a). A constraint of $1.0\text{\AA}$ is the tolerance of the surface extension. The resultant 3948 hits were used as the search database for a refined Unity search, with two hydrogen bonding interactions as additional constraints (donor/acceptor weighting = 0.5): the MTX NA2 donor and Glu 28 Oe2 acceptor pair; and, the Ala 8 backbone amide donor and NADPH nicotinamide O7 acceptor pair (Fig. 4a). This refined search resulted in 212 hits, which were then docked using the Surflex-Dock routine in Sybyl8.0. Docked hits were scored, ranked and visually inspected. We chose 30 of these for *in vitro* enzyme inhibition assays based on their Surflex-Dock score and ranking but also based on their structural heterogeneity (diversity) and their potential for forming hydrogen bonds to active site residues, especially Glu 28. Fifteen of these 30 compounds were available from the DTP Open Chemical Repository, and several milligrams were provided to us as lyophilized samples.

2.4 Inhibition Kinetics

Initially, steady state reaction rates were measured for baDHFR activity in the absence (uninhibited rate) and presence of the NCI compounds at $100\text{ }\mu\text{M}$. All of the compounds save two (28401 and 630361) were soluble in 50 – 100% (v/v) DMSO. Activity was measured as a change in absorbance over time at a wavelength of 340 nm (A_{340}), so as to monitor the disappearance of NADPH. The reaction buffer consisted of 50 mM $\text{KHPO}_4/\text{K}_2\text{HPO}_4$ (pH 7.1), 10 mM β -mercaptoethanol and 1 mM K_2EDTA . To make stock solutions, both the DHF substrate (Sigma-Aldrich) and NADPH cofactor (Alexis Pharmaceuticals) were reconstituted

with the reaction buffer. The concentrations of DHF and NADPH were kept constant for the experiment at 125 μM and 12.5 μM , respectively, and were determined by their absorbance at A_{340} using ϵ values of 7.75 and 6.22 $\text{mM}^{-1} \text{cm}^{-1}$, respectively. The concentration of baDHFR used in the experiment (25 nM) was determined by its absorbance at 280 nm using a theoretically derived ϵ of 27.4 $\text{mM}^{-1} \text{cm}^{-1}$. After incubation of the enzyme with one of the compounds and NADPH for 2 minutes, the reaction was initiated with the addition of DHF and the A_{340} was plotted over the course of 2 minutes. Formal IC_{50} curves were generated for these compounds by testing for baDHFR activity against 5–6 different inhibitor concentrations. All measurements that were used to calculate the IC_{50} were performed in triplicate. The incubations and reactions were performed at RT.

2.5 Dissociation constant (K_d) measurements using fluorescence spectroscopy

Tryptophan fluorescence spectra of baDHFR (0.09 mg/ml (5 μM)) in 15 mM Bis-Tris (pH 5.5) and 500 mM NaCl were recorded using a Jobin Yvon-Spex fluorescence spectrophotometer by exciting the sample with 290 nm light. The protein sample was titrated with increasing concentrations of inhibitor at 25° C. Data were fitted using the one-site binding (hyperbola) equation $Y = B_{\text{max}} * X / (K_d(\text{app}) + X)$ [where B_{max} is the maximum extent of quenching and $K_d(\text{app})$ is the apparent dissociation constant] by nonlinear regression using GraphPad Prism 4.0 software.

3. Results and Discussion

3.1 Overall structure of baDHFR in complex with MTX and NADPH

The entire molecules of $\text{MTX} \cdot \text{NADPH}$ bound to baDHFR were visible in the $2F_o - F_c$ electron density map (Figs. 2a–b). The baDHFR structure has been refined using TLS restraints against data to 2.3 Å resolution to an R_{free} of 27.6% (Table 1). According to a Ramachandran plot and analysis by MolProbity (Davis et al., 2007), the baDHFR $\text{MTX} \cdot \text{NADPH}$ structure adopts reasonable geometry. Compared with our previously solved baDHFR $\cdot \text{MTX}$ binary complex structure, the MTX mode of binding is very similar (Figs. 2b and 3a, b). The main binding energy for MTX is provided by the two carboxylic acids in the *p*-ABA Glu tail that each form salt-bridges with Arg53 and Arg58, respectively (Fig. 2b). Although additional hydrogen bonds are made by the *p*-ABA Glu tail, the N1 and NA2 atoms of the pteridine moiety form two strong hydrogen bonds (both are $\sim 2.8 \text{ \AA}$) with the carboxylate side-chain of the catalytic Glu28 residue. The tip of the pteridine ring of the MTX molecule interacts with the tip of the nicotinamide ring of the NADPH molecule, overlapping so that the closest contact between the two moieties occurs between the nicotinamide NO7 and the pteridine N3 atoms (3.5 Å; Fig. 3b). Hydrogen bonds formed between NADPH and the enzyme are shown in Fig. 2c. Both the N7 and O7 atoms of the carboxamide group of the nicotinamide moiety form hydrogen bonds to the main-chain atoms of Ala 8. The 2'OH and 3'OH of the nicotinamide ribose forms hydrogen bonds with the main-chain amide of Asn20, and the O4 of the ribose forms a hydrogen bond with the hydroxyl group of Thr 125.

The NADPH pyrophosphate bridge sits between the termini of two structurally disparate helices (C and E) and is stabilized by multiple hydrogen bonds to backbone atoms of residues in these helices (Fig. 2c and Supplementary Table 1). The oxygen that bridges the pyrophosphate moiety with the adenine ribose (AO5) forms a hydrogen bond to the backbone amide of Tyr 48. The O4 atom of the adenine phosphoribose forms a hydrogen bond with the carbonyl of Val 63 while the ribose phosphate (off of the 2'O) forms multiple hydrogen bonds with the Arg 45 guanidinium group and the backbone amide of Arg 65. The adenine ring itself only forms two hydrogen bonds and a stacking interaction with Arg 65 (Figs. 2c and 3c).

3.2 Structural comparison with *E. coli* and human DHFR•MTX•NADPH complexes

The MTX inhibitor binds all three DHFRs (the *E. coli* and human enzymes are designated here as ecDHFR and hsDHFR, respectively) almost identically (Figs. 3a–b). The most variable portion of MTX is found at the carboxylic tail. There are several substitutions between baDHFR and hsDHFR that can be exploited for species specific drug design, as detailed in our previous report (Bennett et al., 2007). For the present comparison, we used our baDHFR ternary complex structure and a ternary complex structure of ecDHFR we had previously solved (to 1.5 Å resolution; PDB ID: 3DAU) while for the ternary complex of hsDHFR, we used the structure with PDB ID, 1U72 (1.9 Å resolution) (Cody et al., 2005). The NADPH binds almost identically to DHFR in all three species, with the exception of the adenine ring (Figs. 3a, c). The adenine ring in the baDHFR complex is tilted as much as 85° with respect to the plane of the adenine ring in the ecDHFR and hsDHFR complexes (Fig. 3c). We attribute this difference to be caused by the substitution of Gln 102 in ecDHFR by Leu 104, the equivalent residue in baDHFR. The Gln 102 of ecDHFR forms a hydrogen bond with the adenine ring, an interaction that the Leu 104 in baDHFR cannot make. If the adenine ring in baDHFR was to adopt the *E. coli* conformation, there would be steric clashes with Leu 104. In human DHFR the equivalent residue to Leu 104 is Glu 123, which only makes a van der Waals contact with the adenine ring. On the opposite side of the adenine ring, in both baDHFR and ecDHFR, an Arg residue stabilizes its conformation (Arg 65 for baDHFR, Arg 98 for ecDHFR). In ecDHFR, the Arg 98 residue resides in helix E. Interestingly, although an Arg residue is located at an equivalent position in the sequence on the loop following strand βC for hs and baDHFR, Arg 77 in hsDHFR cannot not form a hydrogen bond with the NADPH adenine ring. The closest contact distance is 4.2 Å. In fact, in the hsDHFR structure, no hydrogen bonds are formed between the adenine moiety and any protein atom. In both baDHFR and ecDHFR, the aforementioned Arg residues make stacking interactions with the adenine ring while hsDHFR does not. Clearly, this is a region of pharmacophore space that should be sampled and exploited in order to gain selectivity, at least between prokaryotic and eukaryotic species.

3.3 In search of dual-site inhibitors using virtual screening

We were interested in developing inhibitors that occupied overlapping sites that partially spanned both the MTX and NADPH binding sites. Our strategy was to use the baDHFR•MTX•NADPH ternary complex to define a pharmacophore that exploited both the MTX and NADPH sites (Fig. 4a). Using the UNITY search option of Sybyl 8.0 software (Tripos Corporation, St. Louis, MO, USA), we were able to rapidly conduct a virtual screen using a library of compounds. The search criteria used in UNITY included three hydrophobic surfaces that were placed on the pteridine ring, the *p*-ABA ring and the nicotinamide ring. The initial UNITY search conducted with the Open NCI database consisting of 260,071 compounds yielded 3948 hits that were ranked based on CScore. CScore is a consensus scoring program that evaluates the results from four different scoring functions which calculate affinities for receptor-ligand interactions and determines an overall score for the binding of a particular receptor-ligand pair (as run within Sybyl 8.0; Tripos Corporation). The set of scoring functions that CScore utilizes are well established: the Docking score (Meng et al., 1992), the Gold score (Jones et al., 1995), the PMF score (Muegge and Martin, 1999; Muegge et al., 1999) and ChemScore (Eldridge et al., 1997). They are all applied individually, and then ranked from 0 to 5 (from worst to best docked candidates) according to the CScore consensus analysis. In the next step, we made a customized database of compounds that consisted of the nearly 4000 hits. We then included two hydrogen bonds as constraints in addition to the three hydrophobic surfaces in the original search criteria- one is formed between the NA2 atom of the pteridine ring of MTX and Glu 28, and the second is formed between the O7 atom of the nicotinamide ring and the backbone amide of Ala 8 (Figs. 2b–c, 4a). The library of nearly 4000 compounds was then subjected to a further UNITY search with these new criteria, yielding 212 hits. The 212 hits were then subjected to Surflex-Dock (Jain, 2003), a docking routine found within the

Sybyl8.0 suite. Surflex-Dock ranks the hits based on multiple docking criteria, which include terms for hydrophobic packing, bump penalties, polar attractive forces (dipole-dipole and hydrogen bonding contacts), repulsive (electrostatics), entropy, and solvation (which compares the potential hydrogen bonds formed between the ligand and receptor versus those formed between the ligand and solvent) (Jain, 2003;Pham and Jain, 2006). All 212 hits were visually inspected, and a subset of 30 compounds was chosen based on scoring and diversity. Further, we only chose compounds that were shown to form a hydrogen bond with the catalytic Glu 28. Although we requested for the 30 compounds, we were only able to obtain 15 of them (Table 2) from the Open Chemical Repository of the NIH/NCI Developmental Therapeutics Program (<http://dtp.nci.nih.gov>), a diverse collection of > 140,000 small molecules.

3.4 Determining inhibition of baDHFR for potential dual-site compounds identified by the in silico screen

The 15 compounds were tested for the inhibition of baDHFR using the canonical spectroscopic assay at A_{340} to monitor the disappearance of NADPH (Bennett et al., 2007). Initially, we tested inhibition of baDHFR activity by the 15 compounds at an inhibitor concentration of 100 μ M. Only two compounds, designated 1357 (6-amino-3-pentadecylphenyl β -D-glucoside) and 373265 (2-(3-(2-(hydroxyimino)-2-(pyridine-4-yl)-6,7-dimethylquinoxalin-2-yl)-1-(pyridine-4-yl)ethanone oxime) by the NCI library (Figs. 1, 4d–e), showed significant inhibition of baDHFR activity at this concentration. We further characterized these by determining their IC_{50} values (Figs. 4b–c): compound 373265 possesses an IC_{50} of 74 μ M while compound 1357 is ~3 times more potent, with an IC_{50} of 16.5 μ M.

We attempted to derive the K_d for each of the compounds using fluorescence spectroscopy. Previously, the binding of MTX to *E. coli* DHFR was shown to decrease tryptophan fluorescence due to quenching of Trp 22 in ecDHFR (Appleman et al., 1988), and the same change in Trp fluorescence upon ligand binding has been observed by others (Dunn and King, 1980). In principal, binding at the substrate and the NADPH binding sites can be evaluated by fluorescence spectroscopy (Appleman et al., 1988). Unfortunately, due to spectral overlap from the inherent fluorescence of the two compounds, only 373265 could be used to measure the apparent K_d for the active site. Importantly, it has no intrinsic fluorescence in the normal range of tryptophan fluorescence emission (~340 nm) but does show enhanced fluorescence at 425 nm possibly because it has absorption in the range of the 360 nm wavelength (see Fig 5a; black squares). Upon titration of 373265, the intrinsic tryptophan fluorescence of baDHFR (red squares in Fig. 5a) is quenched (the pink, blue and green squares in Fig. 5a). Analysis of the binding curves (Fig. 5b) yielded an apparent dissociation constant [$K_{d(app)}$] of $5.329 \pm 0.559 \times 10^{-6}$ M, or 5.3 μ M. It is important to mention here that our IC_{50} measured for the 373265 inhibitor against DHFR (74 μ M) is in moderate agreement with the K_d .

3.5 Docking models of the two inhibitory compounds, 1357 and 373265

The Surflex-Dock routine provides enzyme-ligand docked complexes that can be useful for investigating the potential mode of binding (Jain, 2003; Pham and Jain, 2006). Fig. 4d shows the baDHFR active site with compound 1357 bound at the baDHFR•MTX•NADPH binding sites. Compound 1357 was initially synthesized in the 1950's as an anti-tuberculosis agent (Latham et al., 1951). The docked conformation of 1357 has its glucoside ring spatially overlapping with the pteridine ring, while the 6-amino-3-decylphenyl ring overlaps with the nicotinamide ring, and the aliphatic chain turns about the ribose moiety of the nicotinamide ring and passes through the *p*-ABA-Glu of MTX (Fig. 4d; Supplementary Fig. 1a). A complete set of interactions made by 1357 with baDHFR is provided in Table 3. All the hydrogen bonds are made by baDHFR to the glucoside ring containing 5 hydroxyls: several to the carboxyl side-chain of Glu 28 and one to the backbone carbonyl of Phe 96. The pentadecyl aliphatic chain branched off of the 6-amino phenyl moiety forms multiple van der Waals contacts with

hydrophobic side chains of several baDHFR residues: Ala 50, Leu 21, Ile 51, Phe 96, Leu 29, Leu 55, and the aliphatic portion (C γ) of the Lys 33 side chain. This long, aliphatic chain protrudes from the MTX pteridine-NADPH nicotinamide binding site and into a relatively hydrophobic region near where the *p*-ABA-Glu tail would bind for MTX (Supplementary Fig. 1a).

Our docking model proposes that compound 373265 also binds at partially overlapping sites where MTX and NADPH binds (Fig. 4e; Supplementary Fig. 1b). A complete listing of interactions made between 373265 and baDHFR is listed in Table 4. One of the pyridine rings occupies a site that partially overlaps with the N7 and O7 atoms of the nicotinamide ring (the carboxamide group) and with the pteridine ring while the other pyridine ring protrudes further into the NADPH binding pocket and nearly overlaps perfectly with the nicotinamide ribose. The N1 atom of this dimethylquinoxaline ring forms a hydrogen bond with Asn 47. The 6,7-dimethylquinoxaline ring moiety docks into the C9-N10-*p*-ABA binding site of MTX. In contrast to 1357, this compound is proposed based on its docking to form several hydrogen bonds with baDHFR residues. This is exemplified by the ethanone oxime and hydroxyimino groups (-CH₂-C=N-OH) that connect the pyridine rings to the dimethylquinoxaline ring; these form hydrogen bonds to the carbonyl groups of Met 6, Ile 15 and Asn 20. The pyridine that overlaps with the nicotinamide ribose potentially forms hydrogen bonds to the N δ 2 atom of Asn 19, the carbonyl of Lys 46 and the backbone amide of Asn 47. It should be noted that, as docked, this compound form hydrogen bonds with neither the Ala 8 carbonyl nor the Glu 28 carboxylate, the donor-acceptor constraints implemented in the refined UNITY search. The pyridine overlapping the carboxamide group of the nicotinamide only comes within van der Waals contact distance to both the Ala 8 and Glu 28 (3.8 and 4.2Å, respectively).

4. Conclusions

We determined the X-ray structure of baDHFR in a ternary complex with MTX and NADPH. With this structure, a comparison of the substrate (DHF) and cofactor (NADPH) binding sites between baDHFR and the human enzyme is afforded. Coupling this with findings from our previous work (the baDHFR•MTX binary structure) (Bennett et al., 2007), two major differences in the binding sites can be noted. First, a sequence insertion in the hsDHFR causes a Pro residue (Pro 61) to be adjacent to the MTX *p*-ABA site- this position is a Gly in baDHFR (Gly 52). Previously, it was shown that bulky groups substituted off the pteridine•*p*-ABA linker and the *p*-ABA itself may clash with Pro 61 of hsDHFR whereas they should bind baDHFR without steric interference (Bennett et al., 2007). Now, with the NADPH binding site defined, a second potential pharmacophore region can be exploited to enhance species selectivity and to help evade host resistance. In the baDHFR ternary structure, the adenine ring adopts a rotamer conformation that is severely tilted relative to the same moiety in a deposited hsDHFR structure (1U72) (Cody et al., 2005). An Arg residue (Arg 72) stabilizes this conformation- the equivalent residue in hsDHFR, Arg 77, adopts a rotamer that bends away from the adenine ring. In fact, packing of hsDHFR residues around this region of the cofactor is fairly poor relative to baDHFR, suggesting affinity for therapeutics with similar moieties in similar conformations would be comparatively lower. These two sites, adjacent to the substrate *p*-ABA and the cofactor adenine ring, could be useful in the design of baDHFR-selective dual site inhibitor. Additionally, by extending the pharmacophore space to include two sites, we may be able to design drugs that can overcome host mechanisms of binding site mutagenesis and thus avoid the possibility of adapted resistance.

In order to develop dual-site inhibitors with potential clinical significance, we used the baDHFR•MTX•NADPH complex to conduct virtual screening using UNITY within the Sybyl8.0 suite using the NCI library. The screen was further refined with hydrogen bond restraints yielding a subset of 212 hits. The subset of 212 hits was subjected to further selection

using Surflex-Dock within Sybyl8.0. Thirty compounds were chosen as the best candidates based on Surflex-Dock scores, and *a priori* knowledge of DHFR ligand binding. We were able to obtain 15 out of the thirty compounds from the NCI, and of the 15, two compounds (1357 and 373265) inhibited baDHFR with IC₅₀ values of 16 and 74 μM, respectively. The apparent K_d was determined by fluorescence spectroscopy to be 5.3 μM. The future work would involve kinetically and crystallographically defining these compounds as definite dual-site inhibitors. Further, we concomitantly will use these compounds as leads for further optimization for the development of more potent baDHFR dual-site inhibitors. Neither are derivatives of traditional antifolates, an advantage being that these structurally and chemically distinct compounds possibly represent the first leads of two new classes of DHFR inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DHFR	Dihydrofolate Reductase
ba	<i>Bacillus anthracis</i>
ec	<i>Escherichia coli</i>
hs	<i>Homo sapiens</i>
MTX	Methotrexate
p-ABA	<i>para</i> -aminobenzoic acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
DHF	Dihydrofolate
IC₅₀	Inhibitor Concentration at 50% enzymic rate inhibition

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Appendix

Appendix A. Supplementary data

A table listing the contacts between baDHFR and NADPH and a figure showing the electrostatic surface representation of baDHFR active site with compound 1357 and 373265 bound.

Fig. 1. Chemical structures of MTX, NADPH, DHF, compounds 1357 and 373265. For clarity, only the atom names are provided for the moieties of MTX, NADPH and DHF that bind within the baDHFR active site.

Fig. 2. Crystal structure of (a) *Bacillus anthracis* (ba) DHFR in a ternary complex with MTX and NADPH. DHFR is shown as a cartoon representation. $2F_o - F_c$ electron density for MTX (magenta) and NADPH (dark green) is shown contoured at 1.2σ . (b) and (c) Schematic representation of the (d) MTX and (e) NADPH binding sites in baDHFR, as generated by LIGPLOT (Wallace et al., 1995).

Fig. 3.

Characterization of the MTX and NADPH binding sites. (a) Superposition of ba (green), ec (magenta) and hs (cyan) DHFRs. The structures are displayed as α traces. Labeled numbers correspond to the four major contiguous insertion regions in the hsDHFR sequence, not found in the baDHFR and ecDHFR sequences (Bennett et al., 2007). Two views are shown, related by a 90° turn about the y-axis. The hsDHFR 61–64 insertion, seen in the left-hand panel, protrudes into the MTX *p*-ABA binding site of prokaryotic DHFRs including baDHFR and can be exploited in species-selective drug design, as described in (Bennett et al., 2007). (b) The nicotinamide and pteridine rings of NADPH and MTX, respectively, partially overlap in the active site. The closest interligand atoms that contact each other occur between the nicotinamide O7 and the pteridine N3 atoms; the contact distances are 3.5 Å, 3.8 Å and 3.7 Å in baDHFR, ecDHFR and hsDHFR, respectively. (c) The adenine ring of NADPH in baDHFR is severely tilted ($\sim 85^\circ$) relative to the position of this same moiety in ecDHFR and hsDHFR. In ba and ecDHFR, functionally conserved Arg residues form stabilizing hydrogen bonds to the NADPH adenine; however, the Arg residue in hsDHFR is only close enough to the adenine to form van der Waals interactions.

Fig. 4.

In silico design of a pharmacophore against the MTX and NADPH binding sites of baDHFR and *in vitro* inhibition assays. (a) The pharmacophore was designed to have 5 interaction features as constraints. Three of them are hydrophobic, which all are aromatic rings: the diaminopyrimidine ring of the MTX pteridine ring, the p-ABA ring of MTX and the nicotinamide ring of NADPH. (b) and (c) Curves used for determining IC₅₀ values against baDHFR for (b) compound 1357 and (c) compound 373265. (d) Docking of compound 1357 in the baDHFR active site. (e) Docking of compound 373265 in the baDHFR active site. To demonstrate that both compounds dock into both the substrate and cofactor binding sites, MTX and NADPH are shown from the baDHFR crystal structure. In (d) and (e), carbons are colored

green for compounds 1357 and 373265, magenta for MTX, and cyan for NADPH. Residues from baDHFR that form hydrogen bonds with the docked compound are shown in red. For clarity, moieties of the NADPH distant from the active site are not shown.

Fig. 5.

Tryptophan fluorescence quenching of baDHFR due to the binding of compound 373265. (a) Tryptophan fluorescence spectra of compound only (black squares) and baDHFR (0.09 mg/ml (5 μM) in 15 mM Bis-Tris (pH 5.5) containing 500 mM NaCl) at various μM concentrations of 373265 (0 μM, red squares; 4, pink; 10, blue; and 40, green). (b) Variation of the extent of fluorescence quenching ($F_0 - F/F_0$, where F_0 and F are the fluorescence intensities at 342 nm in the absence and presence of compound 373265) of 0.09 mg/ml (5 μM) of DHFR as a function of 373265 concentration.

Table 1
X-ray crystallographic statistics for the Bacillus anthracis DHFR•MTX•NADPH crystal

baDHFR•MTX•NADPH	
Unit cell: a, b, c(in Å)	81.9, 43.2, 67.9
Unique angle (in °)	$\beta = 119.3$
Space group	C2
Resolution range 15–2.3 Å (2.35 – 2.3 Å)	
Total reflections	342281 (1302)
Unique reflections	8332 (668)
Completeness %	93.8 (76.1)
R_{sym}, % *	14.3 (29.6)
I/σ	10.4 (3.0)
Multiplicity	3.2 (2.1)
R_{work}/R_{free}, % †	23.7/27.7
No. of atoms	
Protein	1282
Ligands	80
Solvent	36
Average B-factors, Å²	
Protein	44.9
Ligand	37.0
Solvent	47.0
Deviations from ideality ‡	
Bond lengths, Å	0.017
Bond angles, °	1.98

* $R_{\text{sym}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \bar{I}(hkl)|}{\sum_{hkl} \sum_i I_i(hkl)}$

† **R_{work} and R_{free}** = $\frac{\sum |F_o| - |\sum F_c|}{\sum |F_o|}$, where F_o and F_c are the observed and calculated structure factor amplitudes. For the calculation of R_{free}, 5% of the reflection data were randomly selected and omitted from refinement.

[‡]As reported from the final refinement cycle of Refmac5 (Murshudov et al., 1997).

Table 2
Refined docking/scoring statistics (Surflex-Dock) and baDHF_R *in vitro* inhibition results.

NCI Ligand ID*	Formula	MW (g/mol)	Docking Score	Rank	Inhibition at 100 uM
215272	C ₂₄ H ₂₇ NO ₄	393	8.55	4	No
382104	C ₂₂ H ₂₅ N ₃ O ₄	395	7.75	14	No
380544	C ₂₂ H ₂₆ N ₆ O ₇	486	7.35	19	No
151840	C ₂₃ H ₂₇ N ₃ O ₃	393	7.35	20	No
2320	C ₂₁ H ₄₀ O ₄	357	7.13	27	No
7396	C ₂₁ H ₄₀ O ₅	373	6.72	40	No
132100	C ₂₁ H ₃₅ N ₅ O ₂	390	6.68	42	No
630372	C ₂₂ H ₂₇ N ₅ O ₂ S	426	6.61	46	No
622389	C ₂₃ H ₂₂ N ₆ O ₅	462	6.35	54	No
1357	C₂₇H₄₇NO₆	482	5.94	75	Yes
179482	C ₂₂ H ₄₄ O ₅	389	5.28	99	No
373265	C₂₄H₂₂N₆O₂	426	4.78	117	Yes
372040	C ₂₀ H ₃₂ N ₆	357	3.9	161	No

* Two of the received compounds (28401 and 630361) were not miscible in DMSO and, thus, were not tested.

Table 3
Residues within hydrogen bonding distance of docked compound 1357[†]

1357 moiety	Residue, atom	Hydrogen bonding distance (Å)
Glucoside ring		
C ₆ hydroxyl	Glu 28, Oε1	2.5
C ₁ hydroxyl	Glu 28, Oε1	2.9
C ₁ hydroxyl	Glu 28, Oε2	3.4
C ₃ hydroxyl	Phe 96, O	3.1

[†]For Tables 3, 4 and Supplementary Table 1, the definition for a hydrogen bond is the interaction between two polar opposite atoms that are 2.4 – 3.5 Å distant from each other, with distances lesser than 3.0 Å being characterized as a strong hydrogen bond and distances greater than 3.0 Å being characterized as a weak hydrogen bond.

Table 4
Residues within hydrogen bonding distance of docked compound 373265

373265 moiety	Residue, atom	Hydrogen bonding distance (Å)
2-Hydroxyimino		
OH	Ile 15, O	3.2
OH	Asn 20, O	3.4
OH	Leu 21, N	3.4
2-pyridine		
N4	Asn 19, Nδ2	3.4
N4	Lys 46, O	3.3
N4	Asn 47, N	3.1
6,7-dimethylquinoxalin		
N1	Asn 47, Oδ1	3.1
Ethanone oxime		
OH	Met 6, O	3.4