A Molecular Docking Strategy Identifies Eosin B as a Non-active Site Inhibitor of Protozoal Bifunctional Thymidylate Synthase-Dihydrofolate Reductase*


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Protozoal parasites are unusual in that their thymidylate synthase (TS) and dihydrofolate reductase (DHFR) enzymes exist on a single polypeptide. In an effort to probe the possibility of substrate channeling between the TS and DHFR active sites and to identify inhibitors specific for bifunctional TS-DHFR, we used molecular docking to screen for inhibitors targeting the shallow groove connecting the two active sites. Eosin B is a 100 μM non-active site inhibitor of Leishmania major TS-DHFR identified by molecular docking. Eosin B slows both the TS and DHFR reaction rates. When Arg-283, a key residue to which eosin B is predicted to bind, is mutated to glutamate, however, eosin B only minimally inhibits the TS-DHFR reaction. Additionally, eosin B was found to be a 180 μM inhibitor of Toxoplasma gondii in both biochemical and cell culture assays.

Electrostatic channeling is a mechanism proposed based on the crystal structure of bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR)‡ from Leishmania major that would enable negatively charged dihydrofolate produced at the TS active site to be handed-off along a series of solvent-exposed lysine and arginine residues to the DHFR active site, where it is converted to tetrahydrofolate (1).

In protozoal parasites, the enzymes TS and DHFR exist as a bifunctional enzyme on a single polypeptide chain. The crystal structure of L. major TS-DHFR revealed a shallow, basic residue-rich groove connecting the two enzyme active sites (1). Based on this structural finding, it has been proposed that the negatively charged dihydrofolate (H₂folate) produced at the TS active site is electrostatically channeled to the DHFR active site, where it is converted to tetrahydrofolate (H₄folate) without equilibration in bulk solvent (1, 2). The shallow groove region is unique to bifunctional TS-DHFR enzymes; however, how channeling occurs and its physiological significance has not been completely established. We used two concurrent approaches to address this issue: the first being mutagenesis of solvent-exposed basic residues thought to play key roles if the channeling behavior is electrostatic in nature. Our second approach, presented here, is to attempt to physically obstruct channeling and/or domain-domain communication. In the case of trypanothione synthase, where substrate channeling occurs through a hydrophobic tunnel, mutation alone was sufficient to obstruct channeling (4). With TS-DHFR our approach was to identify small molecules that may bind in various areas within the shallow electrostatic groove and to examine their ability to inhibit enzymatic activity as well as parasite growth in cell culture.

In an effort to discover an inhibitor that would bind in the unique shallow groove region of the bifunctional TS-DHFR enzyme, we turned to molecular docking screens of the Available Chemicals Directory (ACD) data base. Such a strategy has been previously used to discover novel inhibitors of monofunctional TS, where a competitive inhibitor found by screening with the program NWU DOCK (5–7) was used as a novel scaffold compound for in-parallel, solid-phase synthetic elaboration, generating an analog with a Kᵢ of 1.3 μM (8). In addition, a preliminary DOCK screen produced a 900 μM non-competitive TS active-site inhibitor: a subsequent similarity search yielded derivatives with Kᵢ values of less than 10 μM (9). More broadly, both monofunctional TS and DHFR enzymes have been the foci of intense structure-based design efforts and are considered model enzymes for such projects (10–12).

An unusual aspect of this project was the targeting of sites in the bifunctional TS-DHFR enzyme located in regions remote from the active sites. Enzyme active sites have traditionally served as the basis of structure-based drug discovery because they represent well defined and highly functionalized targets. By contrast, the shallow groove connecting the TS and DHFR active sites in bifunctional enzymes presents a particular
challenge to structure-based efforts, including molecular docking. Successful targeting of the shallow groove region connecting the two active sites and forming the putative channel could produce novel and more specific therapies for protozoal diseases including toxoplasmosis and drug-resistant malaria.

Several docking searches were performed against subregions of a surface area of ~20 × 25 Å thought to define the central region of the electrostatic channel in L. major TS-DHFR (Fig. 1A). This area includes the basic residues Lys-66, -67, -72, -73, -282 and Arg-64, -283, and -287, believed to be involved in shuttling the intermediate substrate, dihydrofolate, between the two catalytic sites (1). Four subregions of the putative channel were screened against the 152,571 compounds in the 1995.2 version of the ACD of commercially available chemicals. From these screens, 14 compounds were purchased for testing as inhibitors of TS-DHFR (Table I).

We report here that eosin B, predicted by DOCK to bind in the shallow groove region of the putative channel including Arg-283 in the TS domain and Glu-151 in the DHFR domain (Fig. 1A), inhibits the bifunctional enzyme. Eosin B (4'-5'-dibromo-2'-7'-dinitrofluorescein) is a halogenated xanthene dye (see Fig. 1A and Table I) whose spectrophotometric properties have been taken advantage of to measure the protein concentration at low pH (1–3) (13). Whereas related halogenated fluorescein derivatives are thought to be nonspecific inhibitors of several enzymes, acting through an aggregation-based mechanism, extensive kinetic characterization suggests that eosin B is inhibiting the bifunctional enzyme specifically (14).

**EXPERIMENTAL PROCEDURES**

**Computational Work**

All docking calculations were performed with the December 1999 development version of NWU DOCK, a version derived from UCSF DOCK 3.5. This version incorporates the ensemble method of ligand flexibility for up to 2000 conformations per molecule (5). The polar and non-polar close contact limits used in the steric grids were 2.3 and 2.6 Å (15). The AMBER united atom charge set, distributed with Delphi, was used for all receptor electrostatic calculations. CHEMGRID was used to calculate a van der Waals potential for the enzyme using standard potentials (16). Chemical labeling was used (17) in the matching calculation. This involves labeling site positions or atoms by chemical properties to speed the docking calculation. Here, five labels were employed: positive, negative, hydrogen bond donor, hydrogen bond acceptor, and neutral. All water molecules and counterions were removed from the receptor model.

We used the L. major TS-DHFR coordinates provided to us by Dr. David Matthews (1). A dielectric of 2 for the protein interior (18) and 78 (19) for the water phase were used in the DelPhi calculations. The internal and external dielectrics in the hydration calculation were also set to 2 and 78. In the DelPhi calculation the probe size was set to 1.4 Å. Atomic van der Waals radii for the protein and the ligand were taken from Rashin (19). In the Delphi calculation, the ionic exclusion radius was set to 2 Å and the ionic molarity was set to 0.1 M. The proper values of ligand and protein dielectrics, probe, van der Waals, and ionic radii are active areas of research; we have not tried to optimize these terms.

In the receptor potential calculation, three-step focusing (20) was used with protein containment iteratively set to 20, 60, and 90% with a 65.3 Å lattice.

All data base searches used the same 152,571 molecule subset of the 1995.2 release of the ACD (21). These molecules were selected based on our ability to calculate partial atomic charges (16) and included most of the molecules in the ACD-3D. Partial atomic charges were calculated by
Molecular Docking Identifies a TS-DHFR Inhibitor: Eosin B

the method of Gasteiger and Marsili (22). Ligand solution corrections were calculated using HYDREX (15).

The shallow groove region between the TS and DHFR-binding sites was explored using sets of spheres to describe the channel. Spheres were prepared starting from the sphen program and filtered through the cluster program (16, 23), and then edited by hand using Midas (24). 37 receptor spheres were used. The mean number of orientations per molecule of eosin B, ACID or TMPD(p)), 0.5, scored 14.2 kcal/mol in run 7, placing its rank as 198 of 152,571 molecules. The best energy score in the same run was 37 kcal/mol.

Chemicals

All buffers and other reagents employed were of the highest chemical purity. Millipore ultrapure water was used for all solutions. CH2H4folate and CH2H4folate were purchased from Schircks Laboratories (Switzerland). H4folate was synthesized by reduction of folic acid with sodium borohydride. Tryptitium-labeled H4folate and CH2H4folate were synthesized using tritiated folic acid as a starting material: [3',5',7,9-3H]folic acid was obtained from Moravek Biochemicals (Brea, CA). Tryptitium-labeled H4folate was chemically prepared from the reduction of folate by sodium hydrosulfite (25). Tryptitium-labeled H4folate was prepared enzymatically: tritium-labeled H4folate was converted to tritiated H4folate by L. major TS-DHFR + NADPH (DHFR reaction), and condensed with formaldehyde to form CH2H4folate. The natural (6R)-1-Ch2H4folate enantiomer was purified by DE-52 anion exchange chromatography (Whatman) and used exclusively in the studies. CH2H4folate and CH2H4folate solutions were stored as argon-purged vials at ~90 °C. NADPH and dUMP were purchased from Sigma; the concentration of NADPH was determined by using a molar extinction coefficient of 6220 M−1 cm−1 at 340 nm. Eosin B was purchased from ABCR (A Better Choice for Research Chemicals, Karlsruhe, Germany); fluorescein and phenolphthalein were purchased from Sigma.

Enzymology

The clone of the wild-type bifunctional TS-DHFR enzyme from L. major was a generous gift from C.-C. Kan and D. Matthews, then at the cluster program (16, 23), and then edited by hand using MidasPlus (24). The sphere set used contained 57 spheres and then rotated manually to more appropriate orientations using Sybyl (Tripos, St. Louis, MO). Positions of some protons were positioned in the protein database file for L. major DHFR to more appropriate orientations using the Swiss Protein Database website. The program NWU-Docking was used to find the desired orientations, as confirmed by nucleic acid sequencing, was used to transform C. 14,094

steady-state Experiments

The reactions were initiated by mixing the 15-fluorochrome solution (0.1–1 μl enzyme; 2× reaction buffer: 1 ml EDTA, 50 mM MgCl2, 50 mM Tris, pH 7.8, saturating NADP and dUMP, and eosin B in MeSO or MeSO alone as control) with the 15 μl of CH2H4folate. 4% of the final reaction volume was reserved for eosin (1 ml eosin B, final) or MeSO (control). The enzyme solution was reacted with 15 μl of substrate: 100–200 μl tritiated CH2H4folate (~20,000 dpm). In all cases, concentrations of enzyme and substrates cited in the text are those after mixing. The reactions were terminated by quenching with 67 μl of 0.78 N KOH to give a final concentration of 0.54 N KOH (2).

Rapid Chemical Quench Experiments

The rapid chemical quench experiments were performed using a Kinetik RFQ-3 rapid chemical quench apparatus (Kinetik Instruments, Austin, TX). Fresh stocks of 25 μl eosin B in MeSO were made prior to each experiment. The final reaction volume was 30 μl; 4% of this was reserved for eosin B (1 ml eosin B, final) or MeSO (control). The single enzyme turnover reaction was initiated by mixing the 15 μl of enzyme solution (enzyme + 2× reaction buffer) with the tritiated substrates (15 μl, approximately 20,000 dpm); in all cases, concentrations of enzyme and substrates cited in the text are those after mixing. The TS-DHFR single enzyme turnover reaction was monitored by addition of tritiated CH2H4folate to enzyme + NADPH and dUMP. The DHFR reaction was monitored by addition of tritiated H4folate to enzyme + NADPH. The enzymatic reactions were terminated by quenching with 67 μl of 0.78 N KOH to give a final concentration of 0.54 N KOH (for more details, see Liang and Anderson (2)). The rate constants for individual single turnover rapid chemical or burst quench experiments were estimated by comparing the data to the single exponential or burst curve using the curve fitting program, Kaleidagraph.

HPLC Analysis—Tritiated products of the rapid quench experiments were quantified by HPLC (high performance liquid chromatography) in combination with a radioactivity flow detector. The HPLC separation was performed using a BDS Hypersil C18 reverse phase column (250 × 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 ml/min. An isoteric separation using a solvent system of 10% methanol in 180 mM triethylammonium bicarbonate, pH 8.0, was employed. The elution times were as follows: H4folate, 9 min; H3folate, 18 min; CH2H4folate, 20 min. The HPLC effluent from the column was mixed with liquid scintillation mixture (Monoflow V, National Diagnostics) at a flow rate of 5 ml/min. Radioactivity was monitored continuously using a Flo-One radioactivity-flow detector (Packard Instrument Co., Inc.). The analysis system was automated using a Waters 712B WISP (Milford, MA) autosampler.

T. gondii TS-DHFR Homology Model

A homology model of T. gondii TS-DHFR was built using the Swiss Protein Database program in conjunction with the Swiss Model homology modeling link available at the Swiss Protein Database website. The C-terminal 315 amino acids (residues 295–610) and residues 115–166 were modeled using the protein database file for the L. major TS-DHFR structure. The N-terminal 52 amino acids were modeled using the protein database file for Pneumocystis carinii DHFR, which is the highest homology DHFR relative to the N-terminal portion of the T. gondii DHFR domain for which a structure was available.

Evaluation of the Ability of Eosin B to Inhibit T. gondii Replication in Cell Culture

Cell Lines and Culture Conditions—Chinese hamster ovary cells were grown as monolayers at 37 °C in a humidified atmosphere of 5% CO2 in α-minimum essential medium supplemented with 2 μg/mL glutamine and penicillin/streptomycin (100 units/ml per 100 μg/ml).

Parasite Cultivation—The RH strain tachyzoite of T. gondii was used and maintained in vitro culture in human foreskin fibroblast cells as described previously (29).

Determination of Parasite Viability—Chinese hamster ovary cells were seeded in triplicate at a density of 2 × 105 cells/ml in a 24-well plate, allowed to attach for 24 h, and then infected with T. gondii. Parasites were cultured were synchronized by removal of parasites that had not yet invaded 4 h after their inoculation into confluent cells. After incubation with various concentrations of eosin B for 24 h, T. gondii viability was evaluated 24 h postinfection by measurement of [3H]uracil incorporated into the parasite nucleic acids. Briefly, 1 μCi of radiolabel was added to each well for 2 h before the monolayers were fixed with trichloroacetic acid, rinsed, and counted as described (30).

RESULTS

Molecular Docking Against TS-DHFR—The program NWU-DOCK (5–7), a derivative of DOCK 3.5 (16, 23) was used to screen 152,571 compounds of the ACD 1995.2 data base for molecules complementary to the shallow groove, putative channeling region of protocoloza TS-DHFR. To prepare the site for docking, all water and ion molecules were removed. Protonation of the receptor residues and water molecules was done with Sybyl (Tripos, St. Louis, MO). Positions of some protons were then rotated manually to more appropriate orientations using MidasPlus (24). The sphere set used contained 57 spheres and was obtained from reclustering spheres obtained from the sphen program, part of the DOCK 3.5 package (17). Force field and electrostatic grids were calculated with CHEMMGRID (16).
The 14 DOCK hits tested, along with the ACD code, two-dimensional structure, and DOCK energy score of each. All inhibitors were tested by rapid chemical quench at a concentration of 1 mM; 50 μL *L. major* TS-DHFR using the same set of reaction conditions as detailed for eosin B. An "*" indicates that upon initial characterization, inhibition was observed at a drug concentration of 1 mM, as compared with a Me2SO control. One compound, 2,5,7-trinitro-9-oxo-9H-fluorene-4-carboxylic acid, elicited complex kinetic behavior, appearing to inhibit in a time-dependent and NADPH-dependent manner. Also included in the table are the structures of two subsequently tested eosin B analogs, fluorescein and phenolphthalein.

<table>
<thead>
<tr>
<th>Compound Name/ Chemical Formula/ ACD Code</th>
<th>Structure</th>
<th>DOCK Score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 2-((3,3-dimethyl-2-oxobenzyl)thio)-6-methyl-5,5-dioxo-5,6-dihydro-5-λambda-6-benzoiopyrimido[4,5-E][1,2]thiazine-8-carboxylate</td>
<td><img src="image1" alt="Structure" /></td>
<td>-17.1</td>
</tr>
<tr>
<td>2,5,7-nitro-9-oxo-9H-fluorene-4-carboxylic acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>-14.7 (*</td>
</tr>
<tr>
<td>N-(4-Nitrophenylsulfonyl)-L-phenylalanine</td>
<td><img src="image3" alt="Structure" /></td>
<td>-15.7</td>
</tr>
<tr>
<td>METHYL 7-FLUORO-2-(METHOXYMETHYL)-5-OXO-5H-CHROMEN[2,3-B]PYRIDINE-3-CARBOXYLATE</td>
<td><img src="image4" alt="Structure" /></td>
<td>-16.1</td>
</tr>
<tr>
<td>5-ISATINSULFONIC ACID, SODIUM SALT HYDRATE</td>
<td><img src="image5" alt="Structure" /></td>
<td>-13.1</td>
</tr>
<tr>
<td>2,2-dimethyl-4-oxo-7-(phenylsulfonyl)oxy)-3,4-dihydro-2H-chromen-5-yl-benzene-1-sulfonate</td>
<td><img src="image6" alt="Structure" /></td>
<td>-18.7</td>
</tr>
<tr>
<td>4-hydroxy-3,5-dinitrobenzoic acid</td>
<td><img src="image7" alt="Structure" /></td>
<td>-13.7</td>
</tr>
<tr>
<td>N-O-NPS-glutamine</td>
<td><img src="image8" alt="Structure" /></td>
<td>-15.3</td>
</tr>
<tr>
<td>3-aminio-3-(G-nitrophenyl)propionic acid</td>
<td><img src="image9" alt="Structure" /></td>
<td>-17.3</td>
</tr>
<tr>
<td>2-(4-(4-(4-aminophenyl)(thio)-3-nitrobenzyl)amino)propanoic acid</td>
<td><img src="image10" alt="Structure" /></td>
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<tr>
<td>N3-(4-chloro-2-[[2-(phenylhydrazino)carbonyl]phenyl]-2-phenoxyisocyanuramide</td>
<td><img src="image11" alt="Structure" /></td>
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<tr>
<td>2-(methoxycarbonyl)benzo[b]thiophen-3-yl-2-phenoxyisocyanate</td>
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<tr>
<td>5-benzzyloxy- DL-tryptophan</td>
<td><img src="image13" alt="Structure" /></td>
<td>-12.2</td>
</tr>
<tr>
<td>Eosin B</td>
<td><img src="image14" alt="Structure" /></td>
<td>-14.2</td>
</tr>
<tr>
<td>Fluorescein</td>
<td><img src="image15" alt="Structure" /></td>
<td></td>
</tr>
<tr>
<td>Phenolphthalein</td>
<td><img src="image16" alt="Structure" /></td>
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</table>
Molecular Docking Identifies a TS-DHFR Inhibitor: Eosin B

Fig. 2. Eosin B dose response. A, steady-state time courses were completed in the presence of 0.1 μM L. major TS-DHFR, 200 μM CH₂H₄folate, and 0 μM (Me₂SO control), 62.5, 125, or 250 μM eosin B. An IC₅₀ value of 100 μM was obtained by plotting the rates of the steady-state reactions versus eosin B concentration. B, steady-state time courses with 1.5 μM T. gondii TS-DHFR, 500 μM CH₂H₄folate, and varying concentrations of eosin B yielded an IC₅₀ of 180 μM. C, in cell culture assays, an eosin B concentration of 180 μM also reduced T. gondii replication inside host cells by 50%, as expressed by counts/min of radiolabeled incorporated into parasites.

Species dependence of eosin B inhibition on the TS-DHFR, TS, and DHFR reactions; and effects of eosin B on the L. major R283E mutant

Data are reported as percent activity remaining: rate of the reaction in the presence of 1 mM eosin B divided by the rate with Me₂SO alone. All values where an error margin is included represent the average results from 2 to 3 paired time courses. The TS-DHFR reaction is not applicable to the E. coli monofunctional enzymes.

<table>
<thead>
<tr>
<th>Species/enzyme</th>
<th>TS-DHFR</th>
<th>TS</th>
<th>DHFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. major</td>
<td>24 ± 3%</td>
<td>23 ± 3%</td>
<td>35 ± 0%</td>
</tr>
<tr>
<td>T. gondii</td>
<td>22 ± 3%</td>
<td>29%</td>
<td>28 ± 8%</td>
</tr>
<tr>
<td>E. coli</td>
<td>NA*</td>
<td>37 ± 0%</td>
<td>91 ± 0%</td>
</tr>
<tr>
<td>L. major R283E</td>
<td>80%</td>
<td></td>
<td>30%</td>
</tr>
</tbody>
</table>

* Not applicable.

In the case of R283E, 80% of the R283E TS-DHFR activity remains in the presence of 1 mM eosin B, but note that R283E has 40% L. major wild-type TS-DHFR activity. R283E has 100% wild-type DHFR activity.

Transient Kinetic Analysis—Transient kinetic analysis was used to obtain a more detailed characterization of the action of eosin, including effects on the single enzyme turnover rates of the TS and DHFR reactions individually, as well as in the bifunctional TS-DHFR reaction, and to look for a build-up of dihydrololate that may suggest evidence of impaired channeling. Whereas steady-state kinetic analysis is an indirect method from which one can infer information about the rate-limiting step of an enzymatic reaction, transient kinetics allows one to directly measure individual steps in a kinetic pathway as well as to define the reaction kinetics of intermediate formation. Transient kinetics has several advantages for investigation of substrate channeling because, in principle, this technique enables one to directly monitor chemical catalysis at each active site as well as the transit of the putative intermediate from one active site to another (32, 33).

Single enzyme turnover experiments, which measure the rate of the chemical conversion of substrate to product at the active site under conditions where enzyme concentration is sufficiently high that substrate binding is not rate-limiting, were performed using a rapid chemical quench apparatus. For each single enzyme turnover experiment, full time courses for L. major in the presence and absence of 1 mM eosin B were completed in duplicate, along with t = 0 and t = 60 s controls. Because eosin B was dissolved in dimethyl sulfoxide, an equivalent amount of Me₂SO (4% of final volume) was added to control reactions. This concentration of Me₂SO has no effect on the single-turnover reaction rates (data not shown).

To monitor the DHFR reaction, the bifunctional TS-DHFR enzyme (50 μM) was preincubated with a saturating concentration of NADPH (500 μM) and then mixed with a limiting amount of radiolabeled H₂folate (14 or 15 μM). To monitor the TS reaction, the bifunctional TS-DHFR enzyme (50 μM) was preincubated with a saturating concentration of dUMP (500 μM) and then mixed with a limiting amount of radiolabeled CH₂H₄folate (10 or 12.5 μM). The bifunctional TS-DHFR single enzyme turnover experiment was set up similar to that for TS, except that saturating NADPH (500 μM) was added as well as dUMP. All reported concentrations are final, after mixing.

Eosin B was found to inhibit both the TS and DHFR reactions, but TS was inhibited more strongly (Table II). As predicted, the inhibition of the bifunctional reaction by eosin B is
equivalent to inhibition of TS alone because the rate of product formation by TS is significantly slower than that of DHFR in *L. major*. The data are presented in Table II as the percent of activity remaining; the ratio of the rate constant obtained in the presence of 1 mM eosin B divided by that with Me2SO alone. In experiments examining the bifunctional TS-DHFR reaction, no dihydrofolate accumulation above background was observed.

To address the question of whether the inhibition observed in the single enzyme turnover experiments described above was because of competition with substrates at the active site, the effect of varying the substrate concentration was examined. When either 3 or 62 μM CH2H4folate was reacted with 250 μM *L. major* TS-DHFR in the presence of 1 mM eosin B, 33–40% activity remained. In the case of the DHFR reaction, equivalent reaction rates were obtained when 4 or 40 μM H2folate was reacted with 100 μM *L. major* TS-DHFR in the presence of 1 mM eosin B. The fact that a 20-fold change in CH2H4folate concentration and a 10-fold change in H2folate concentration had no effect on level of inhibition by eosin B at enzyme concentrations where binding of substrate is not rate-limiting, implies that eosin B binds outside of both the TS and DHFR folate binding pockets.

**Species Variations in Inhibition by Eosin B**—Because one long-term goal of this research is to develop a therapy specific for protozoal bifunctional TS-DHFR, eosin B was tested against TS-DHFR from an evolutionarily divergent but clinically relevant sporozoan protozoa, *T. gondii*, and against *E. coli* monofunctional TS and DHFR for comparison (Table II). Single turnover experiments were set up as detailed above for *L. major* (50 μkat enzyme, 1 mM eosin B or 4% Me2SO, 500 μM dUMP and/or NADPH, with ~10 μM radiolabeled CH2H4folate or H2folate).

The bifunctional TS-DHFR as well as the individual TS and DHFR reactions of *L. major* and *T. gondii* were similarly inhibited by addition of 1 mM eosin B. The *E. coli* TS reaction was slightly less inhibited than that of *L. major*. The striking finding was, however, that whereas ~35% DHFR activity remains with both bifunctional enzymes in the presence of 1 mM eosin B, *E. coli* DHFR is almost entirely unaffected by 1 mM eosin B (91% activity remaining). Whereas the TS enzyme is highly conserved across species, the relatively low sequence conservation in DHFR makes sequence alignments inconclusive. Structural alignment of *L. major* with a homology model of *T. gondii* TS-DHFR, however, reveals a similar overall structure in the region of Glu-151, with *T. gondii* residue Asp-146 located very near to and in the same orientation as Glu-151. By contrast, an examination of the aligned crystal structures reveals that the loop structure of *E. coli* in the vicinity of Glu-151 is significantly different from that in *L. major*, and the closest acidic residue, Asp-90, is oriented in a different direction from Glu-151.

**Effects of Eosin B on the *L. major* R283E Mutant**—Because eosin B was predicted by the docking program to interact with Arg-283 (R283) in the TS domain of *L. major*, an analogous experiment was performed (Fig. 1B). R283E was tested against the charge reversal mutant, R283E. Whereas mutation of Arg-283 alone produced an enzyme with only 40% of wild-type TS-DHFR activity, addition of 1 mM eosin B only inhibited the mutant enzyme slightly such that >80% activity remains. This sharply contrasts what was seen with the wild-type enzyme where, under the same reaction conditions, 1 mM eosin B strongly inhibits the wild-type enzyme such that ~20% activity remains (Fig. 3A). Furthermore, the Arg-283 → Glu substitution had very little effect on ability of eosin to inhibit the DHFR reaction, consistent with the docking prediction (Fig. 3B). Effects of eosin B on the *L. major* R283E mutant are summarized in Table II.

**Comparative Enzyme Inhibition by Eosin Analogues**—To evaluate the contributions of structural components of eosin B to its inhibitory properties, eosin B was compared with 2 analogs: fluorescein and phenolphthalein (see Table I). Fluorescein, the unliganded precursor to eosin B lacking the halogen and nitro groups, was found to be ~8-fold less potent than eosin B itself. At a concentration of 1 mM eosin B, the rate of the TS-DHFR reaction was only 25% that in the absence of inhibitor. In contrast, at 1 mM fluorescein no inhibition was observed; and at 4 mM, a rate that was 50% of the TS-DHFR reaction rate with no inhibitor added could be achieved (Fig. 4).
observed with 1 mM fluorescein. was more significant on the apicomplexan parasite, inhibitor (data not shown), however, the sensitivity of eosin B parasites multiply in a parasitophorous vacuole, (624,000). It was seen that an eosin B concentration of 180/H11021 allow the passive bidirectional diffusion of small molecules eosin B in that it contains phenolic and benzoic acid moieties concentration of 400/H9262 notable because parasite host cells were unaffected up to a drug ured by [3H]uracil incorporation (Fig. 2). This is remarkable because parasite host cells were unaffected up to a drug concentration of 400 μM (assayed by [3H]thymidine incorporation). To establish that the inhibitory effects of eosin B in cell culture were because of effects on the folate pathway, the ability of leucovorin (folic acid) to “rescue” inhibited cells was assessed. The finding that 10 μM leucovorin reverses the inhibition of replication induced by eosin B provides strong evidence that TS-DHFR is the target of eosin in T. gondii.2

**DISCUSSION**

Molecular docking was used to target the shallow groove connecting the TS and DHFR active sites of the L. major bifunctional enzyme; the surface originally hypothesized to form an electrostatic channel for the substrate intermediate, dihydrofolate. Eosin B, predicted by NWU DOCK to interact with Arg-283 in the TS domain and Glu-151 in the DHFR domain of the L. major bifunctional enzyme, was found to have an IC50 value of 100 μM. Although such a level of inhibition is modest by drug standards, first round lead compounds from virtual screening often inhibit in this range; modification can improve these leads by several orders of magnitude (3, 17, 36, 37). Because targeting the non-active site of a molecule for inhibition requires a more in-depth understanding interplay of the mechanism and structure than targeting the active site, we sought to address these issues prior to attempting generation of more potent and specific inhibitors.

A non-active site TS-DHFR inhibitor could act by interfering with channeling of dihydrofolate from TS to DHFR, protein conformational changes constituting TS-DHFR domain-domain communication, or both. As discussed below, a number of experiments did provide evidence that eosin B is a non-active site inhibitor, capable of inhibiting both TS and DHFR. Because the proposed binding site for eosin B is located in the shallow groove region containing the putative electrostatic channel, one might anticipate that a small molecule bound in this region, or mutations that alter charge, might interfere with the transit of dihydrofolate from the TS to the DHFR active site. Accordingly, one might predict that the kinetics would reflect a build-up of the dihydrofolate intermediate in solution. Using a transient kinetic analysis, a single enzyme turnover experiment examining the TS-DHFR reaction did not, however, show a difference in dihydrofolate accumulation in the presence or absence of eosin B. As a likely alternative to inhibition of substrate channeling, eosin B may exert its effect by interfering with domain-domain communication, or the series of protein conformational changes induced by ligand binding at one active site that affects activity at the active site of the other enzyme.

The finding that eosin B only marginally inhibits the L. major R283E charge reversal mutant supports the suggestion that eosin B does in fact, interact with Arg-283, a residue located in the shallow groove targeted with the docking program. Whereas phenolphthalein is known by x-ray crystallography to bind to the TS active site, it inhibits R283E to the same degree as wild-type L. major TS-DHFR. This observation suggests that the binding sites for eosin B and phenolphthalein are distinct, and is not surprising because phenolphthalein is most similar to the cyclic form of eosin B while the docking predictions and our experimental conditions employ the open form. Nonetheless, the finding indicates that the decreased ability of eosin to bind R283E is unlikely to be because of a remote effect, such as change in the active site conformation, as a result of the non-active site mutation. The fact that TS is a highly conserved enzyme and position 283 is completely conserved as a basic residue (arginine or lysine) across enzyme species could account for the similar TS inhibition observed with the bifunctional and monofunctional enzymes tested.

Conversely, whereas there is high structural similarity between L. major and a homology model of T. gondii TS-DHFR in

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Molecular Docking Identifies a TS-DHFR Inhibitor: Eosin B

the region of Glu-151, limited structural conservation exists with *E. coli* DHFR and the closest acidic residue, Glu-90, is oriented in a different direction from Glu-151; possibly explaining the poor inhibition of eosin in the *E. coli* enzyme. Efforts are underway to definitively elucidate the site of binding of eosin through co-crystallization of eosin B with *L. major* and *T. gondii* TS-DHFR.

Whereas Arg-283 and Glu-151 appear to make key contacts with the hydroxyl groups on eosin B, comparative inhibition by the scaffold molecule, fluorescein, predicts that the hydroxyl groups are not the only functional groups on the molecule that are important for binding. The hydroxyl groups are present on both the scaffold molecule and eosin B, but eosin B is an 8-fold better inhibitor of *L. major* TS-DHFR than is fluorescein. This suggests that the 4',5'-dihromo and 2',7'-dinitro groups on eosin B also promote binding, either directly or indirectly, by increasing the hydrophobicity of the inhibitor. Reviewing the structure, the one functional group that does not appear to participate in energetically favorable interactions is the 5'-bromine. This bromine does, however, lie close to a hydrophilic pocket near the conserved basic loop in the DHFR domain, and therefore may be ideally positioned for modification. Understanding structural characteristics that contribute to inhibition by eosin B should allow for structure-based design of analogs with greater potency and specificity for the bifunctional enzyme in the future.

The evaluation of the mode of action of eosin using spectroscopic techniques is difficult because, at the concentrations necessary to see inhibition, the dye absorbs light making fluorescence and absorbance studies unfeasible because of inner filter effects. Transient kinetic studies indicate that eosin B is not competitive with monoglutamyl folate substrates (either CH$_2$H$_2$folate or H$_2$folate). The finding that eosin B inhibits both the TS and DHFR reactions without competing for either folate substrate provides kinetic evidence, supporting the structural predictions of DOCK and results with the R283E mutant, that eosin B binds outside of the active sites of both TS and DHFR.

One aspect of eosin B with which we were concerned was its potential ability to form aggregates in solution that could inhibit many enzymes nonspecifically. Indeed, there is a precedent for fluorinated dyes acting as nonspecific inhibitors (14). Several lines of evidence suggest, however, that eosin B is, in fact, inhibiting *L. major* TS-DHFR specifically. First, IC$_{50}$ values for aggregate-based inhibition are very sensitive to changes in enzyme concentration. With eosin B, on the other hand, steady-state inhibition was unaffected by 10-fold increases in the concentration of *L. major* TS-DHFR (from 0.1 to 1 µM enzyme). If eosin B were forming large aggregates capable of binding up enzyme, the same concentration of aggregates should have been formed in either case, as the same eosin B concentration was used; but a much greater percent inhibition would be observed at low enzyme concentration. Instead 10% inhibition was seen at both enzyme concentrations. Second, a point substitution, Arg-283 → Glu, diminished the inhibition of eosin by over an order of magnitude, consistent with the docking prediction of a classical 1:1 complex being formed in this region. Finally, in the pre-steady-state kinetic rates, a ratio of inhibitor:enzyme of 20:1 was used, whereas aggregation-based nonspecific inhibitors appear to require ratios of over 10000:1 (inhibitor:enzyme). Nonspecific inhibition therefore seems unlikely to explain the activity of eosin versus TS-DHFR.

Evidence that eosin B is specifically targeting TS-DHFR has been further obtained by cell culture experiments with *T. gondii*. *T. gondii* replication is reduced by 50% at a drug concentration of 180 µM, similar to the IC$_{50}$ of eosin in steady-state biochemical assays with *T. gondii* TS-DHFR protein. Furthermore, growth inhibition induced by eosin B is reduced by administration of leucovorin, a chemical that is used as an antidote to compounds that block the conversion of folic acid to folinic acid. Additionally, concern over potential toxicity related to nonspecific inhibition by eosin B is lessened by the finding that no adverse effects were observed in rats fed diets containing up to 2% eosin B, leading to FDA approval of eosin B for use in drugs and cosmetics.

Differences in folate metabolic pathways as well as drug uptake or stability may account for the poor inhibition of *L. major* by eosin B in cell culture. *L. major* is known to encode P-glycoprotein family members including MDR and MRP homologs, and also contains a large vacuole and acidic compartments not present in other parasites. Eosin B uptake and localization within the various parasites will be a topic of future experimentation.

In principle, non-active site binding regions offer unique opportunities to discover specific inhibitors. Often, however, such sites lack the tightly defined geometrical and functional constraints of active sites and as such are difficult to target. Here we were able to use molecular docking to target the shallow groove region between the TS and DHFR active sites in the *L. major* bifunctional enzyme. Several lines of evidence suggest that eosin B binds outside of the two active sites. The effects of the Arg-283 → Glu point substitution are, moreover, consistent with the docking prediction. Whereas mutation of basic residues in the shallow groove region, including Arg-283, does not appear to affect domain-domain communication, or at least TS catalysis; eosin B binding in this region affects both TS and DHFR activity, presumably by inducing or preventing key conformational changes. This, coupled with the fact that no build-up of dihydrofolate was observed, suggests that eosin B exerts its effects on TS and DHFR via interference with domain-domain communication rather than electrostatic channeling.

Furthermore, comparative inhibition by eosin B analogs has yielded structural information that should allow for design of more potent and specific inhibitors of protozoal TS-DHFR and could produce novel therapies specific for parasitic infections, including toxoplasmosis and malaria, particularly drug-resistant malaria. Eosin B has already been shown to be an inhibitor of *T. gondii* cell culture at concentrations where host cells are unharmed. As such, findings with eosin B represent an important step toward establishing proof-of-principle that the non-active site, shallow groove region of the bifunctional TS-DHFR enzyme can serve as a molecular target that when inhibited, results in parasite death.

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REFERENCES


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