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Virtual screening reveals allosteric inhibitors of the *Toxoplasma gondii* thymidylate synthase-dihydrofolate reductase

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Abstract

The parasite *Toxoplasma gondii* can lead to toxoplasmosis in those who are immunocompromised. To combat the infection, the enzyme responsible for nucleotide synthesis thymidylate synthase-dihydrofolate reductase (TS-DHFR) is a suitable drug target. We have used virtual screening to determine novel allosteric inhibitors at the interface between the two TS domains. Selected compounds from virtual screening inhibited TS activity. Thus, these results show that allosteric inhibition by small drug-like molecules can occur in *T. gondii* TS-DHFR and pave the way for new and potent species-specific inhibitors.

Keywords

Virtual Screening; Thymidylate synthase; Dihydrofolate reductase; *Toxoplasma gondii*

The parasite *Toxoplasma gondii* (Tg) is responsible for toxoplasmosis. Across the world, about 1/3 of the population may be infected by the organism.¹ Infection can occur when an individual is immunocompromised during HIV infection, in those having received organ transplants, and in women who are pregnant. Under these conditions, the parasite can form cysts in the brain that can eventually result in depression, anxiety, and schizophrenia² in addition to fatal toxoplasma encephalitis and birth defects. To combat the infection, molecular targets are needed for drug therapy.

One suitable target is the bifunctional enzyme thymidylate synthase-dihydrofolate reductase (TS-DHFR), responsible for nucleotide synthesis. Thymidylate synthase catalyzes the transfer of a methylene group from methylene-tetrahydrofolate to dUMP to create dTMP necessary for DNA replication.³ Conserved arginines facilitate substrate binding by transversing the dimer interface and contacting the dUMP molecule in the adjacent monomer.⁴ Proper orientation of the TS monomers is therefore required for catalysis. Peptides targeting the dimer interface in the human TS have been recently reported as well as the crystal structure of human TS in the apo-active site form with the peptide bound at a

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Supplementary data

Full details on the experimental section as well as supplemental tables and figures. This material is available free of charge via the Internet at <http://www.elsevier.com>.

cavity in the TS/TS interface (PDB ID: 3N5E).⁵ The structure of bifunctional TgTS-DHFR in the presence of dUMP and the folate inhibitor PDDF has also been solved (PDB ID: 4EIL).⁶ There is no obvious interface cavity in the apo-active site human TS without the peptide bound nor in the liganded Tg or human TS structures.^{4a, 6–7} This structural information suggests that the peptide in human TS causes the domains to move apart from one another, creating a pocket in which the peptide is able to bind.

This conformational change upon nucleotide binding is significant in part due to its pharmacological relevance. While human and TgTS share a large degree of sequence and structural conservation, several differences in the primary sequence of TS/TS interface residues the two enzymes exist (Supplementary Figure 1). Given that one amino acid substitution is sufficient to significantly alter conformational changes in human TS, these sequence differences could cause unique molecular motions for each version of TS, allowing for the design of selective, allosteric inhibitors.^{7–8} Peptides that bind to the interface between the apo-dUMP TS domains of both Tg and human TS disrupt the organization of the TS/TS interface and thus reduce TS activity.^{5, 9} Recent results suggest that the conformational changes that take place in unliganded human TS to allow for peptide binding could also occur TgTS.⁹ We therefore reasoned that small drug-like molecules could bind at the TS/TS interface in *T. gondii*. However, the unliganded structure of TgTS-DHFR with a peptide inhibitor at the TS/TS interface has not yet been solved. We therefore employed two strategies to model the putative allosteric binding site at the TgTS/TS interface formed by conformational changes that take place upon peptide binding to the apo-dUMP enzyme (Figure 1). The first strategy superimposed the monomers from the TgTS-DHFR crystal structure on the monomers of the peptide-bound human TS structure. The superimposed model mimicked the shift in monomers relative to each other that takes place upon peptide binding (Figure 1a). The superposition of TgTS-DHFR on human TS revealed an RMSD of 1.052 Å, indicating that the organisms share a high degree of structural homology.

The second strategy created a homology model using the amino acid sequence of TgTS (GenBank accession code: AAB00163) and the structure of peptide-bound human TS (Figure 1b). The program SWISS-MODEL was used to generate the homology model.¹⁰ This strategy modeled the shift in monomers relative to each other as well as specific loop movements that take place upon peptide binding. Superimposing the homology model on the TgTS crystal structure provided an RMSD of 0.86 Å, indicating that most of the model matched the solved structure. The portions of the model that differed most significantly from the structure were near the predicted peptide-binding site at the TS/TS interface.

For the superimposed model, we used the SiteMap function of the Schrodinger suite Glide software to locate a large continuous hydrophobic patch in the TS/TS interface pocket (Figure 2).¹¹ This region was explored computationally using CASTp¹² and LIGSITE.¹³ This analysis revealed that the cavity between the two TS subunits in the superimposed model had a volume of 104.3 Å³ compared to the 160 Å³ peptide-binding pocket in human TS. The cavity in the superimposed model was used for docking 14,400 compounds in the screening library Maybridge Hitfinder, a subset of the ZINC database containing drug-like screening compounds.¹⁴ This approach has been used to successfully target allosteric pockets in bifunctional TS-DHFR from other species.¹⁵ A selection criteria was used where the top hundred hits from the initial run were then screened against the TS active site rather than the allosteric site. The purpose of the selection criteria was to find compounds with the highest Glide XP score difference. Out of this new list, based on the ranking, the compounds suggested to be more likely bind to the allosteric site preferentially over the TS active site were chosen for inhibition assays (Supplementary Table I). A total of 10 compounds for the two different virtual screens were purchased for evaluation. To determine whether the selected compounds inhibit TS activity, the TgTS-DHFR was preincubated with the selected

compounds from virtual screening and the TS reaction was initiated with the addition of methylene tetrahydrofolate and dUMP. All 5 compounds (**1–5**) showed ~50% TS inhibition at 100 μ M (Figure 2D).

Next, we employed the same virtual screening strategy using the homology model. SiteMap located a hydrophobic pocket at the TS/TS interface of the homology model at a similar location as the superimposed model, but with a different shape for the target area selected for molecular docking (Figure 3).¹¹ After performing a virtual screen using Glide and the Maybridge Hitfinder library, the five compounds with the highest Glide score were selected for *in vitro* inhibition studies (Supplementary Table II). Of these, **7**, **9**, and **10** displayed at least 50% inhibition of TS activity at 100 μ M (Figure 3D).

Previous studies using peptides targeting the TS/TS interface revealed that peptide binding was selective for the apo-dUMP form of the enzyme.^{5a, 9} We therefore wished to determine whether the compounds tested displayed a preference for the apo-enzyme relative to the dUMP-bound enzyme. We followed up on compounds that displayed approximately 50% or greater inhibition at a concentration of 100 μ M by testing for this kinetic phenotype. Compounds **1** and **10** displayed the greatest degree of selectivity for the apo-enzyme (Figure 4). We therefore determined the IC₅₀ values for these two compounds against the apo-enzyme and the dUMP-bound enzyme. Preincubating TS with dUMP increased the IC₅₀ of **1** from 74 $\pm$ 22 μ M to 190 $\pm$ 52 μ M and increased the IC₅₀ of **10** from 51 $\pm$ 11 to 142 $\pm$ 38 μ M. This suggests that these compounds bind preferentially to the enzyme in its apo-state conformation, a novel finding for small-molecule inhibitors of TS.

We next tested compounds **1** and **10** for inhibitory promiscuity. We used a previously described assay for assessing promiscuity of small molecule inhibitors by determining the potency of compounds **1** and **10** when preincubating TgTS-DHFR with 0.1 mg/mL bovine serum albumin (BSA) before addition of the inhibitor, which prevents nonspecific aggregation.¹⁶ If small molecules inhibit the enzyme by nonspecifically inducing aggregation, the addition of BSA is therefore predicted to lessen the potency of the compounds. On the other hand, if the small molecules are specifically inhibitory for TS, the addition of BSA should not impact their potency. We found that inhibition by compound **1** was significantly decreased in the presence of BSA (Supplementary Figure 2A), demonstrating that this molecule could be acting promiscuously. However, the IC₅₀ of compound **10** was only slightly increased in the presence of BSA (Supplementary Figure 2B). It is possible the effects on the IC₅₀ in the presence of BSA were due to hydrophobic protein-inhibitor interactions that sequesters small molecules. This was confirmed by evaluating a known specific TS inhibitor, 2'-naphthyl-9H-pyrimido[4,5-b]indole, that is also hydrophobic,¹⁷ as the IC₅₀ values was also slightly increased in the presence of BSA (Supplementary Figure 2C). This suggests that compound **10** has a mechanism of inhibition independent from inducing aggregation, indicating that the compound is specific for TS. Steady-state mode of inhibition studies revealed that compound **10** displayed mixed inhibition of TS activity with respect to dUMP binding and noncompetitive inhibition with respect to CH₂H₄F binding (Supplementary Figure 3). These modes of inhibition are indicative allosteric binding. Compound **10** did not have any impact on DHFR activity (data not shown), further validating that its mechanism of inhibition is not based on nonspecific aggregation.

Since these compounds lacked selectivity over the human TS (data not shown), a next step in the design process will be addressing the species selectivity. Structural studies with the TgTS-DHFR and human TS are underway to facilitate this process. We have recently shown that potent, selective inhibitors targeting the TS active site of the TgTS-DHFR can be developed.¹⁷ To conclude, due to the high degree of structural and sequence homology, two

models of the peptide-bound state of TgTS were made using the peptide-bound human TS structure. Virtual screening of an allosteric site at the TS interface of these models revealed the first instance of small molecule, allosteric inhibitors of TgTS activity targeting the TS/TS interface. These proof of concept studies indicate that targeting this allosteric site for inhibition can offer a new direction to explore for the design and optimization of novel and more potent inhibitors that can be used to treat toxoplasmosis.¹⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Models of *T. gondii* TS/TS interface binding site. (A) Superimposed model created by removing the DHFR domains from the *T. gondii* TS-DHFR crystal structure and superimposing the *T. gondii* TS structure on the peptide-bound human TS structure. Dashed lines indicate that the coordinates of the DHFR domains were removed to facilitate the superposition. (B) Homology model of *T. gondii* TS created using the amino acid sequence of TgTS and the peptide-bound human TS structure.

Figure 2.

T. gondii TS/TS interface of the superimposed model. (A) Overall superimposed model of apo-dUMP, peptide-bound *T. gondii* TS shown in surface representation. The two monomers are colored red and salmon. **1** is shown in green sticks. (B) Zoom of the predicted allosteric site where the ligand was docked using Glide. (C) Cartoon representation of the allosteric site with the inhibitor in the center. Tryptophan 476 from each monomer, shown as sticks, makes hydrophobic interactions with the inhibitor. (D) Inhibition of TS activity by compounds selected from the superimposed apo-dUMP model. Compounds were preincubated with the apo-enzyme and tested at 100 μ M under steady-state reaction

conditions. Experiments were performed in duplicate or triplicate with error bars representing the standard deviation between repeats.

Figure 3.

T. gondii TS/TS interface of the homology model. (A) Overall homology model of apo-dUMP, peptide-bound *T. gondii* TS shown in surface representation. The two monomers are colored red and salmon. **10** is shown in green sticks. (B) Zoom of the predicted allosteric site where the ligand, **10**, was docked using Glide. (C) Cartoon representation of the allosteric site with the inhibitor in the center. Tryptophan 476 from each monomer, is shown in sticks. (D) Inhibition of TS activity by compounds selected from the homology model. Compounds were preincubated with the apo-enzyme and tested at 100 μM under steady-state reaction conditions.

Figure 4.

Apo-dUMP preferential binding of compounds **1** and **10**. *T. gondii* TS activity was measured with varying concentrations of either (A) **1** or (B) **10**. Compounds were mixed with *T. gondii* TS-DHFR either in the apo-enzyme form (blue circles) or following preincubation of the enzyme with dUMP (red triangles). The data were fit to a Michaelis-Menten curve. Data points were performed in duplicate with error bars representing the standard deviation between repeats. Superimposed over each graph is the chemical structure of the compounds tested.