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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.

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 schizophrenia[2] 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 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 Fig. 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 (Fig. 1). The first strategy superimposed the monomers from the TgTS–DHFR crystal structure on the

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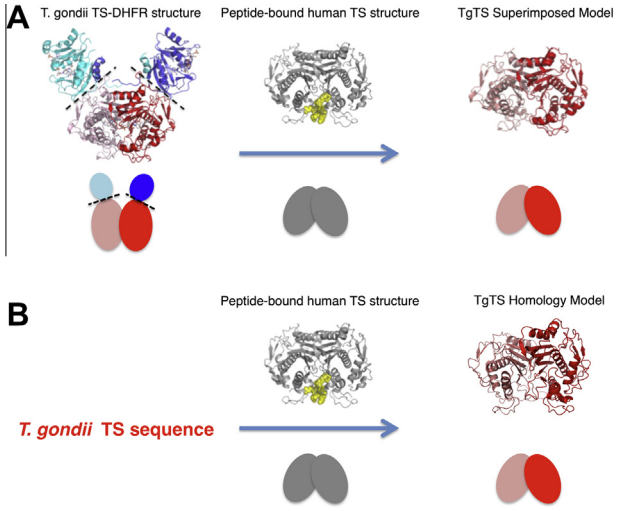

Figure 1. Models of *T. gondii* TS/TS interface binding site. (A) Superimposed model created by removing the DHFR domains from the *T. gondii* TS–DHFR crystal structure and superimposing the *T. gondii* TS structure on the peptide-bound human TS structure. Dashed lines indicate that the coordinates of 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.

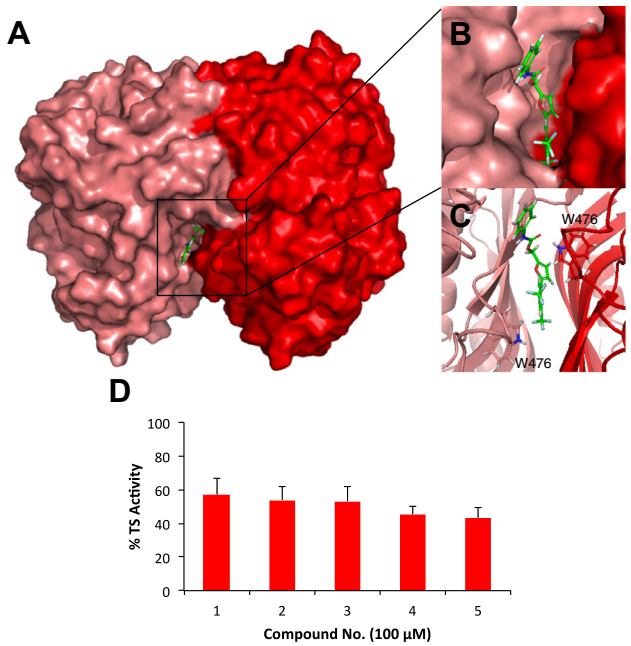

Figure 2. *T. gondii* TS/TS interface of the superimposed model. (A) Overall superimposed model of apo-dUMP, peptide-bound *T. gondii* TS shown in surface representation. The two monomers are colored red and salmon. Compound **1** is shown in green sticks. (B) Zoom of the predicted allosteric site where 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 μM under steady-state reaction conditions. Experiments were performed in duplicate or triplicate with error bars representing the standard deviation between repeats.

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 (Fig. 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 (Fig. 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 (Fig. 2).[11] This region was explored computationally using CASTp[12] and LIGSITE.[13] This analysis revealed that the cavity between the two TS subunits in the superimposed model had a volume of 104.3 $Å^3$ compared to the 160 $Å^3$ 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 1). 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 μM (Fig. 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 (Fig. 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 2). Of these, **7**, **9**, and **10** displayed at least 50% inhibition of TS activity at 100 μM (Fig. 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 μM by testing for this kinetic phenotype. Compounds **1** and **10** displayed the greatest degree of selectivity for the apo-enzyme (Fig. 4). We therefore determined the IC_{50} values for these two compounds against the apo-enzyme and the dUMP-bound enzyme. Preincubating TS with dUMP increased the IC_{50} of **1** from 74 ± 22 to 190 ± 52 μM and increased the IC_{50} of **10** from 51 ± 11 to 142 ± 38 μM. 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