Selective peptide inhibitors of bifunctional thymidylate synthase-dihydrofolate reductase from *Toxoplasma gondii* provide insights into domain–domain communication and allosteric regulation

Mark J. Landau,^{1,2} Hitesh Sharma,¹ and Karen S. Anderson^{1,2*}

¹Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

²Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Received 14 June 2013; Accepted 17 June 2013

DOI: 10.1002/pro.2300

Published online 27 June 2013 proteinscience.org

Abstract: The bifunctional enzyme thymidylate synthase–dihydrofolate reductase (TS–DHFR) plays an essential role in DNA synthesis and is unique to several species of pathogenic protozoans, including the parasite *Toxoplasma gondii*. Infection by *T. gondii* causes the prevalent disease toxoplasmosis, for which TS–DHFR is a major therapeutic target. Here, we design peptides that target the dimer interface between the TS domains of bifunctional *T. gondii* TS–DHFR by mimicking β -strands at the interface, revealing a previously unknown allosteric target. The current study shows that these β -strand mimetic peptides bind to the apo-enzyme in a species-selective manner to inhibit both the TS and distal DHFR. Fluorescence spectroscopy was used to monitor conformational switching of the TS domain and demonstrate that these peptides induce a conformational change in the enzyme. Using structure-guided mutagenesis, nonconserved residues in the linker between TS and DHFR were identified that play a key role in domain–domain communication and in peptide inhibition of the DHFR domain. These studies validate allosteric inhibition of apo-TS, specifically at the TS–TS interface, as a potential target for novel, species-specific therapeutics for treating *T. gondii* parasitic infections and overcoming drug resistance.

Keywords: enzyme kinetics; peptide inhibition; conformational changes; allosteric sites; species-selectivity

Abbreviations: AU, absorbance units; CH₂H₄F, 5,10-methylene-5,6,7,8-tetrahydrofolate; DHFR, dihydrofolate reductase; DNA, deoxyribonucleic acid; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; H₂F, 7,8-dihydrofolate; H₄F, (6R,S)-5,6,7,8-tetrahydrofolate; IC₅₀, half maximal inhibitory concentration; k_{cat} , turnover number; k_{chem} , rate of chemistry; K_d , dissociation constant; K_m , Michaelis–Menten constant; M , molar; mM , millimolar; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; nm, nanometers; nM , nanomolar; s, second; TS, thymidylate synthase; μM , micromolar; V_{max} , maximum velocity.

The authors declare no conflict of interest.

*Correspondence to: Karen S. Anderson, Yale University Medical School, Department of Pharmacology, 333 Cedar St., Rm SHM B350, New Haven, CT 06510. E-mail: karen.anderson@yale.edu

Introduction

Key interactions at protein–protein interfaces constitute important targets for small molecule inhibition because of their specific arrangements and biological importance.¹ The structural variability of these interfaces can allow for the design of inhibitors that distinguish between proteins from different species.² These interfaces therefore comprise possible novel, nonactive, or allosteric target sites for inhibitors of essential enzymes that require specific multimeric organization.

The dimeric enzyme thymidylate synthase (TS) converts deoxyuridine monophosphate (dUMP) to

Figure 1. Schematic of the reactions catalyzed by bifunctional TS-DHFR. (A) TS methylates dUMP to form dTMP using CH₂H₄F as a cofactor. (B) DHFR reduces H₂F to form tetrahydrofolate (H₄F) using NADPH as a cofactor.

deoxythymidine monophosphate (dTMP) using methylene tetrahydrofolate (CH₂H₄F) as a cofactor [Fig. 1(A)].^{3,4} It therefore plays a crucial role in the biosynthesis of DNA and constitutes an important drug target for both anti-cancer and anti-parasitic therapies. TS exhibits half-sites reactivity in which only one monomer is catalytically active at a given time.^{5–8} The coordinated interaction between each monomer of TS plays a role in binding the substrate dUMP and maintaining optimal enzyme catalysis. Thus, proper dimerization of the enzyme is vital for its function.^{5–8}

Unlike human and bacterial TSs, the TS enzyme is physically linked to dihydrofolate reductase (DHFR) in several species of pathogenic protozoa, forming a bifunctional TS-DHFR.^{9–13} DHFR recycles the dihydrofolate (H₂F) formed as a product of the TS reaction into tetrahydrofolate (H₄F) [Fig. 1(B)].¹⁴ Drugs commonly used in the treatment of toxoplasmosis target the DHFR active site.¹⁵ However, the toxicity of these active site inhibitors and the fact that protozoan parasites rapidly build resistance to these compounds underscores the need to design novel therapeutics that specifically target allosteric regions to complement current therapeutic regimens.^{16,17} Previous mutational analyses of different regions of TS-DHFR unique to the parasite have demonstrated the importance of domain-domain communication between the TS and DHFR regions of the enzyme and identified allosteric regions as targets for selective inhibition.^{15,18–22}

Earlier studies with human and *Lactobacillus casei* TS have shown that peptides mimicking specific β -strands at the TS-TS dimer interface can stabilize the apo-enzyme in which each monomer in the dimer is held in conformational “di-inactive” state lacking catalytic activity.^{23,24} Structural studies with the human TS co-crystallized with an interface peptide confirmed peptide binding at the TS-TS interface region.²³ An open question is whether the TS-dimer interface targeted by these peptides can be applied as a strategy to inhibit bifunctional TS-DHFR such as *T. gondii*. Moreover, a more challenging issue is whether this can be accomplished in species-specific manner. Here, we provide the first

example of the use of an interface-binding peptide to interrupt not only the TS activity of the *T. gondii* bifunctional TS-DHFR enzyme but also the distal DHFR catalysis, thereby confirming domain-domain interactions. Most importantly, the interface-binding peptide is specific for *T. gondii* TS-DHFR enzyme relative to the human TS.

A combination of steady-state and pre-steady-state kinetic analyses established that β -strand mimetic interface peptides of the TS-TS dimer interface in the *T. gondii* TS-DHFR inhibit the apo-enzyme in a species-specific manner without affecting the human TS. Fluorescence spectroscopy was employed to monitor conformational changes in the TS domain of the bifunctional *T. gondii* TS-DHFR induced by the presence of dUMP, phosphate, or peptide. Finally, mutational analysis enabled the identification of key residues in the linker region between TS and DHFR that play a role in peptide inhibition of catalysis at the DHFR domain and domain-domain interactions. Taken together, these reveal a new allosteric region in *T. gondii* TS-DHFR and validate the apo-enzyme TS-TS interface as a target for inhibition. These proof-of-concept studies pave the way for the design of small molecules to accomplish selective inhibition of *T. gondii* TS-DHFR and ultimately developing new antiparasitic therapies for toxoplasmosis.

Results

Design of species-selective, TS-dimer interface mimetic peptides for *T. gondii* TS-DHFR

Peptides mimicking the C20 region at the interface of human TS (Leu198 to Gly217) were reported to bind the TS-TS interface and inhibit TS activity.²³ Based upon these studies, the sequences of *T. gondii* TS-DHFR and human TS were aligned to determine the sequence of amino acids in *T. gondii* analogous to the C20 region in human TS [Fig. 2(A)]. The alignment identified a stretch of 21 amino acids (Leu492 to Cys512), herein defined as C21 [Fig. 2(A), in orange] that contained eight residues differing from the human TS C20 sequence. An examination of the *T. gondii* TS-DHFR structure and the TS dimer interface [Fig. 2(B), monomers shown in red and pink]²⁵ confirmed that these residues formed a β -strand-turn- β -strand motif at the TS-TS interface near the dUMP-binding site [Fig. 2(C,D)]. The C21 peptide, composed of amino acids from this region, inhibited *T. gondii* TS activity and induced a concentration-dependent lag in catalysis [Fig. 2(E)]. The unexpected ability of the C21 peptide to induce a lag in the TS reaction was not reported for the interface peptides from human or *L. casei* TS,^{23,24} suggesting that the inhibitory action of the peptide may involve a unique mechanism. It is possible that the observed lag represents a period of time during

Figure 2. Design of TS-TS interface peptide. (A) Sequence alignment of *T. gondii* TS domain (GenBank accession code: AAB00163) and human TS (GenBank accession code: NP_001062) with regions mimicked by the C21 peptide highlighted. (B) The overall structure of *T. gondii* TS-DHFR showing the N-terminal DHFR domain (cyan), the C-terminal TS domain (red), the linker region (purple), and the C21 β -strand-loop- β -strand (yellow-green-blue). The position of TS ligands dUMP (cyan) and the $\text{CH}_2\text{H}_4\text{F}$ analogue PDDF (N^{10} -propargyl-5,8-dideazafofate, magenta) are shown. Dotted purple lines represent portions of the linker missing in the electron density. The TS junctional region of the linker is boxed. (C) Close-up view of C21 β -strand-loop- β -strand showing position of dUMP. (D) Top view of C21 β -strand-loop- β -strand in proximity to dUMP. (E) Absorbance traces of TS steady-state reaction showing lag time and slowed rate caused by incubation with 20 μM (red) or 50 μM (green) C21 peptide compared to the enzyme alone (blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

which the peptide forces the enzyme into an unproductive conformation where ligand binding cannot occur. In this scenario, the enzyme would have to undergo a slow conformational change to regain the ability to bind dUMP and begin the catalytic cycle. The presence of the peptide also reduces the rate of enzyme turnover following the lag. The results therefore indicate that the peptide impairs the ability of the enzyme to bind the active site ligands and catalyze the TS reaction.

Steady-state and pre-steady-state analysis of peptide inhibition

Incubating varying concentrations of the C21 peptide with apo-enzyme *T. gondii* TS-DHFR revealed a potent, concentration-dependent decrease of the steady-state rate of TS catalysis [Fig. 3(A), orange circles]. Interestingly, mixing C21 with *T. gondii* TS-DHFR that had been preincubated with the active site ligand dUMP abolished the inhibitory effect of C21. This suggests that the peptide requires the apo-

enzyme to bind. Importantly, the peptide did not have an effect on the steady-state activity of human TS [Fig. 3(A), blue diamonds] and did not induce a lag (data not shown), demonstrating species selectivity of enzyme inhibition by C21. We also found that C21 inhibited *T. gondii* DHFR, albeit slightly less potently than its inhibition of TS [Fig. 3(B), orange circles]. The C21 peptide had no effect on DHFR activity when the enzyme was preincubated with dUMP and also did not affect human DHFR [Fig. 3(B), blue diamonds]. This analysis indicates that the C21 peptide is specifically binding to the apo-enzyme form of *T. gondii* TS-DHFR and is capable of inhibiting both the TS and DHFR active sites without affecting the human TS or DHFR enzymes.

To determine the sequence dependence of the C21 peptide and identify C21 fragments with inhibitory activity, we tested several shorter fragments of C21 (Table I). The peptide LK mimicked the C-terminal β -strand and displayed weak, species-specific, and apo-selective inhibition of TS activity

Figure 3. C21 inhibition of TS and DHFR activities. The effect of varying concentrations of C21 on either (A) TS or (B) DHFR activity. Orange circles: C21 incubated with apo-enzyme *T. gondii* TS-DHFR; black circles: *T. gondii* preincubated with dUMP and then mixed with C21; blue diamonds: the effect of C21 on (A) apo-enzyme human TS or (B) human DHFR. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but did not influence DHFR. The peptide FS was taken from portion of C21 that contained the last three residues of the C-terminal β -strand, the turn region, and the first three residues of the N-terminal β -strand. The FS peptide displayed more potent inhibition of TS activity than LK and was also both apo-selective and species-specific. FS also demonstrated a slight species-specific inhibition of the DHFR domain. The LR peptide was constructed to mimic the peptide that was co-crystallized with human TS, taken from a portion of the N-terminal β -strand.²³ However, the *T. gondii* LR peptide lacked species selectivity as it also inhibited human TS and DHFR enzymes. The inhibition of monofunctional DHFR by LR demonstrated its general lack of selectivity. Finally, we constructed the peptide DC to mimic the entire N-terminal β -strand. DC inhibited both TS and DHFR active sites and displayed improved species specificity and apo-selectivity compared to the LR peptide but not relative to C21. Thus, these results demonstrate that the full-length C21 peptide is more potent than any of the peptide fragments and the potency, species-specificity, and apo-selectivity of the peptides are sequence-dependent.

We next sought to identify the mechanism of inhibition by these peptides (Fig. S1). None of the peptides had any effect on the steady-state $V_{\max}$ or K_m of either human TS or human DHFR. However, all of the peptides displayed a mixed inhibition pattern against *T. gondii* TS activity and the peptides C21 and DC displayed a noncompetitive inhibition pattern against *T. gondii* DHFR. Taken together, the data confirm that these peptides mimicking the TS–TS interface do indeed bind allosterically.

A more detailed analysis of C21 peptide inhibition, under pre-steady-state conditions, allowed an assessment of its effect on the rate of chemistry of the DHFR reaction for *T. gondii* TS-DHFR. To measure the k_{chem} of the DHFR reaction, a stopped-flow single-turnover reaction was performed in the absence and presence of peptide [Fig. 4(A,B)]. A concentration of 50 μM peptide decreased the k_{chem} of the DHFR reaction from $361 \pm 14 \text{ s}^{-1}$ to $149 \pm 5 \text{ s}^{-1}$. The effect of the peptide on the rate of the overall bifunctional single-turnover reaction to evaluate effects on TS catalysis for *T. gondii* TS-DHFR was determined via rapid chemical quench with in-line radioactivity detection. The presence of 50 μM peptide reduced the rate of consumption of $\text{CH}_2\text{H}_4\text{F}$

Table I. IC_{50} 's, Species-Selectivity, and Apo-Specificity of C21 and Peptide Fragments

Peptide	Sequence	T_g TS IC_{50} (μM)	hTS IC_{50} (μM)	T_g DHFR IC_{50} (μM)	hDHFR IC_{50} (μM)	Apo-specific
C21	LCQFYVNDQKELSCIMYQRSC	41.9 ± 5.3	NI ^a	84.1 ± 17.8	NI	Y
LK	LCQFYVNDQK	778 ± 96	NI	NI	NI	Y
FS	FYVNDQKELS	335 ± 49	NI	27% ^b	NI	Y
LR	LSCIMYQR	180 ± 31	120 ± 21	247 ± 23	247 ± 26	N
DC	DQKELSCIMYQRSC	68.3 ± 7.3	239 ± 38	120 ± 20	18% ^b	Y

^a NI, No inhibition detected at concentrations tested (up to 500 μM peptide).

^b Percent inhibition at 500 μM peptide.

Peptides were considered apo-specific if no inhibition of *T. gondii* TS-DHFR was observed when the enzyme was preincubated with 100 μM dUMP.

Figure 4. Inhibition of pre-steady-state TS and DHFR activities by C21. (A,B) Representative stopped flow traces of *T. gondii* DHFR single-turnover with either (A) 0 μM or (B) 50 μM C21 peptide. (C,D) Bifunctional single-turnover reaction of TS–DHFR tracing either (C) $\text{CH}_2\text{H}_4\text{F}$ consumption or (D) H_4F production with either 0 μM (black) or 50 μM (gray) C21 peptide. No significant buildup of the intermediate product H_2F was detected in either scenario.

from $3.2 \pm 0.4 \text{ s}^{-1}$ to $1.3 \pm 0.2 \text{ s}^{-1}$ and the rate of appearance of the final product, H_4F , from $3.0 \pm 0.3 \text{ s}^{-1}$ to $1.5 \pm 0.2 \text{ s}^{-1}$ [Fig. 4(C,D)]. The rate-limiting step of the bifunctional TS–DHFR reaction is the chemical catalysis for the TS reaction.⁷ This more comprehensive analysis indicates that presence of the peptide slows the rate of chemistry at both the TS and DHFR active sites.

Fluorescence studies to probe molecular mechanism of peptide inhibition

Earlier structural and fluorescence studies with human TS established that the enzyme undergoes conformational switching between active and inactive states dependent on ligand binding.^{26–31} The observation that the peptides were able to inhibit *T. gondii* TS–DHFR in an apo-specific manner, similar to what has been observed for human TS, raised the question of whether *T. gondii* TS might also utilize an analogous conformational switching to couple active and inactive conformational states. Conformational switching involves a change of position of a TS active site loop (residues 181–197 in human cor-

responding to residues 475–491 in *T. gondii*), which contains the catalytic cysteine, Cys489, that would modulate transitions between active and inactive conformations (Fig. S2). This transition is observed by monitoring intrinsic tryptophan fluorescence change upon binding of either dUMP or phosphate. The TS active site loop contains a conserved tryptophan residue (Trp182 in human corresponding to Trp476 in *T. gondii*) that changes position during active–inactive transitions in human TS while the positions of other tryptophan residues are unaffected.³² Binding dUMP transitions the enzyme into the active conformation and changes the position of the active site loop such that a decrease in fluorescence is observed.^{32,33} Binding of inorganic phosphate in place of the dUMP phosphate forces the active site loop into the inactive conformation, which orients Cys489 away from the active site. In this scenario, an increase in fluorescence is observed.^{32,33} Conformational switching between active and inactive states has been observed for human TS, but is reportedly absent for *Escherichia coli* (*E. coli*) and mouse TS.^{32,33}

Figure 5. Monitoring TS conformational changes via fluorescence. (A,B) Changes in fluorescence for (A) human TS or (B) *T. gondii* TS–DHFR upon addition of phosphate or dUMP. (C) Change in fluorescence of *T. gondii* TS when preincubated with dUMP and mixed with phosphate or C21 peptide. (inset: zoomed in view of fluorescence peaks) (D) Change in fluorescence of *T. gondii* TS when incubated with C21 peptide and then mixed with either phosphate or dUMP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Similar to human TS, we found that *T. gondii* TS undergoes conformational switching that causes an increase in fluorescence upon addition of phosphate and a decrease of fluorescence upon addition of dUMP [Fig. 5(A,B)]. A phosphate concentration of 30 mM and a dUMP concentration of 20 μ M were sufficient to maximize the change in fluorescence for the inactive and active states, respectively. Preincubation of *T. gondii* TS with 20 μ M dUMP prevented any significant change in fluorescence upon addition of the C21 peptide, while 80 μ M dUMP was required to prevent a change in fluorescence upon phosphate binding [Fig. 5(C)]. However, when added to *T. gondii* TS in the apo-enzyme state, the C21 peptide caused a decrease in signal which saturated at approximately 30 μ M peptide, indicating a conformational change upon binding [Fig. 5(D)]. The addition of phosphate to the peptide-bound protein did not cause a change in the fluorescence signal, suggesting that the peptide forces the protein into a distinct conformational state that prevents phosphate binding. The addition of 20 μ M dUMP to the peptide-bound protein restored the fluorescence signal of enzyme's active conformation. However, 50 μ M peptide still had an inhibitory effect in the presence of 20 μ M dUMP [Fig. S1(B)]. Therefore, although 20 μ M dUMP seemingly recovers the active conformation of the enzyme, peptide inhibition still occurs. A

concentration of 1 mM dUMP restored approximately 91% of the steady-state TS rate when the enzyme was preincubated with 50 μ M C21 peptide, although a lag time in the reaction of approximately 15 s was still observed (data not shown). This suggests that a sufficiently high concentration of dUMP can indeed outcompete peptide binding and overcome steady-state peptide inhibition but cannot prevent the lag preceding enzyme turnover. The data indicates that dUMP and the peptide bind to distinct conformations of TS, supporting our previous kinetic data demonstrating that the presence of the peptide causes a decrease in the rate of catalysis and an increase in the apparent K_m of dUMP. Plotting the change in fluorescence signal as a function of the concentration of C21 gave a K_d of $23 \pm 8 \mu$ M, near the calculated IC_{50} of $41.9 \pm 5.3 \mu$ M (Fig. S3). These results suggest that *T. gondii* TS undergoes conformational switching similar to human TS and can adopt both active and inactive forms.

To confirm that fluctuations in the position of residue Trp476 were indeed responsible for changes in fluorescence signal, we constructed the mutant W476A. When the fluorescence of the W476A mutant was monitored, it was found that there was no change in fluorescence signal whether phosphate, dUMP, or peptide was added [Fig. S4(A)]. This observation verified that the fluorescence changes seen in

Figure 6. Effect of the TS junctional region of the linker on peptide inhibition and enzyme activity. (A) Close-up of the TS junctional linker region (purple) extending into the N-terminus of the TS domain (red) with interactions targeted by mutagenesis highlighted. (B,C) Steady-state efficiencies of WT and linker junction mutants for (B) TS and (C) DHFR activity. Efficiencies were calculated using the formula $\text{Efficiency} = k_{\text{cat}}/K_m$ and the K_m 's for dUMP (TS) and H₂F (DHFR). (D) Effect of mutations on steady-state DHFR inhibition by C21 and DC. The crossover helix deletion is labeled as $\Delta 291-296$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

wild type were indeed caused by a change of position of Trp476 upon ligand binding. Furthermore, the mutation decreased TS activity approximately 70-fold but did not significantly affect DHFR activity [Fig. S4(B)]. The mutation also significantly increased the K_m of dUMP ($18 \pm 5.5 \mu\text{M}$) compared to that of wild-type ($0.33 \pm 0.7 \mu\text{M}$). Taken together, the data demonstrate that the peptides alter the conformation of the TS active site loop and the Trp476 residue plays a role in maintaining optimal TS activity.

Mutational analysis to investigate domain–domain communication

This study as well as other studies have suggested domain–domain communication for bifunctional *T. gondii* TS–DHFR.²⁵ Therefore, the structure was examined to identify potential mechanisms involved in domain–domain communication, in particular, the TS junctional region of the linker that connects the TS and DHFR domains. Domain–domain communication between the monomers of the bifunctional enzyme has been described with mutational analysis and as a target for species-selective inhibitors in other bifunctional TS–DHFR enzymes.^{18,19,21} The linker region extends from the DHFR of one monomer and donates a helix (termed the crossover helix) that contacts the other monomer [Fig. 2(B)]

near the DHFR active site. Mutations of the crossover helix have been shown to impact the activity of both the DHFR and TS active sites in *Cryptosporidium hominis*²¹ and *T. gondii* TS–DHFR.²⁵

It is unclear how the crossover helix is able to impact the catalytic activity of the distal TS in the *T. gondii* TS–DHFR. As illustrated in Figure 6(A), we identified five residues in the TS junctional region of the linker which extends from the crossover helix into the TS domain (residues 311–320). These residues are unique to *T. gondii* TS–DHFR. Three of these residues (Arg320, Asp329, and Asn336) form contacts between the TS junctional region and the N-terminus of the TS domain directly downstream. In addition, His316 forms contacts with Glu567 in a downstream loop of the TS domain and Phe319 appears to form hydrophobic or base-stacking interactions with Phe231 of the DHFR domain. Phe319 and Phe231 are also near enough to the crossover helix from the other monomer to form hydrophobic interactions with crossover helix side chains. The loop formed by the stretch of residues in the TS junction is the only part of the linker that contacts both the TS and DHFR domains. Three mutants were constructed to identify a possible role of the TS junctional linker region in maintaining optimal activity of both active sites. One construct contained the

Table II. Kinetic Constants of *T. gondii* TS–DHFR and the TS Junctional Region Mutants

	WT	RDN	F319A	HFRDN
K_m dUMP (μ M)	0.33 ± 0.07	3.12 ± 0.41	0.36 ± 0.07	8.00 ± 0.94
TS k_{cat} (s^{-1})	1.18 ± 0.04	1.21 ± 0.07	$0.99 \pm .05$	0.51 ± 0.01
K_m H ₂ F (μ M)	0.15 ± 0.03	0.11 ± 0.02	1.09 ± 0.38	2.10 ± 0.27
DHFR k_{cat} (s^{-1})	3.60 ± 0.22	3.83 ± 0.24	2.02 ± 0.10	2.14 ± 0.06
DHFR k_{chem} (s^{-1})	361 ± 14	512 ± 36	540 ± 23	621 ± 26

mutations R320A/D329A/N336A (RDN), the second one contained the single mutation F319A alone, and the third contained mutations of all five residues identified, H316A/F319A/R320A/D329A/N336A (HFRDN). The effects of these mutations on the catalytic efficiency of the TS and DHFR activities was evaluated as well as whether the presence of the mutations influenced the ability of the C21 and DC peptides to inhibit TS and DHFR activities.

A complete summary of the kinetic parameters for each of the three TS junctional mutants is compared with WT in Table II and the overall catalytic efficiencies for TS and DHFR are shown in Figure 6(B,C), respectively. When the kinetic phenotype of the RDN mutant was determined, it was found that the mutation increased the K_m of dUMP approximately ninefold compared to wild type. The HFRDN mutant increased the K_m of dUMP approximately 25-fold and also reduced the k_{cat} of the TS reaction approximately twofold. As shown in Figure 6(B), both of these mutations significantly reduced the enzymatic efficiency of the TS reaction, but the HFRDN mutant also had a substantial impact on the enzymatic efficiency of the DHFR reaction, particularly on the K_m of H₂F [Fig. 6(B,C); Table II]. Both the RDN and HFRDN mutants also increased the K_m 's of NADPH and CH₂H₄F by approximately twofold compared to WT. The F319A single mutant did not significantly affect TS activity or NADPH binding, but did affect H₂F binding similar to the HFRDN mutant. Interestingly, all three mutants increased the k_{chem} of the DHFR reaction as measured by stopped-flow fluorescence. The HFRDN mutant had the largest change in DHFR k_{chem} with an increase of approximately 70% (Table II).

As shown above, kinetic analysis demonstrated that both the C21 and DC peptides inhibit TS and DHFR activities in a species-specific manner (Table I). To further understand the underlying mechanism of this inhibition, the peptide inhibition for the C21 (orange) and DC (purple) peptides were examined for their ability to inhibit the catalytic activities of three TS junctional mutants to ascertain whether these mutations influenced the inhibition. As illustrated in Figure 6(D), each peptide showed decreased inhibition of the DHFR activity of the HFRDN mutant. The two peptides were comparably potent against the DHFR activities of the wild type and RDN mutants of TS–DHFR. We also examined a crossover helix-

deleted mutant of *T. gondii* TS–DHFR (Δ 291–296) to determine whether the donated crossover helix played a role in DHFR inhibition by the peptides, but the extent of inhibition for this mutant was similar to that of the wild type enzyme. The F319A single mutation had no effect on DHFR inhibition by C21 but did have an intermediate effect on DHFR inhibition by DC. The HFRDN mutant also displayed lessened inhibition by C21 of DHFR chemistry as measured by stopped flow fluorescence [Fig. S5(A)]. Both peptides still potently inhibited the TS reaction of the HFRDN mutant, demonstrating that peptide binding was unaffected by the mutations [Fig. S5(B)].

Discussion

The enzyme TS plays an important role in cellular metabolism and is a target for the treatment of parasitic infections and cancer. In this study, the importance of interactions in the TS–TS dimer interface for enzyme function has been examined with interface mimetic peptides. These data show that TS–TS interface mimetic peptides inhibit the bifunctional *T. gondii* TS–DHFR under both steady-state and pre-steady-state conditions. The TS domain from *T. gondii* undergoes conformational switching between active and inactive forms, the first reported nonprimate TS enzyme to do so.³⁴ Previous studies have demonstrated that the residue Arg163 in human TS stabilizes its inactive conformation and is essential for conformational switching.^{26–29,34} The position of this residue corresponds to Asn457 in the TS domain of *T. gondii* TS–DHFR [Fig. 2(A)]. Our fluorescence data therefore suggest that this asparagine residue could also be capable of stabilizing the inactive state and allowing for conformational switching. In *Plasmodium falciparum* TS–DHFR, the TS residue in the corresponding position is also an asparagine, whereas in *Cryptosporidium hominis* TS–DHFR it is a lysine, in *Leishmania major* TS–DHFR it is a leucine, and in *Trypanosoma cruzi* TS–DHFR it is an alanine. Additional fluorescence experiments revealed that while the *P. falciparum* TS also displayed conformational switching activity, TS from *C. hominis*, *L. major*, and *T. cruzi* are incapable of switching between inactive and active states (data not shown). This further indicates that an asparagine residue in this position can allow for conformational switching in TS from protozoan TS–DHFR while lysine, leucine, and alanine cannot. Future work will examine in

Figure 7. Schematic of the conformational changes of *T. gondii* TS. Apo-TS samples both inactive and active conformations. Adding phosphate transitions the enzyme into an inactive conformation. Adding the interface-mimicking peptide C21 to apo-TS traps the enzyme in an inactive conformation distinct from the phosphate-bound state. Adding dUMP stabilizes the enzyme in the active conformation and rescues TS from the inactive states induced by peptide or phosphate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

detail the role of residue Asn457 in *T. gondii* TS conformational switching.

The presence of a C21 peptide caused a decrease in fluorescence signal upon binding, indicating that the peptide induces a conformational change upon binding TS as illustrated in the model of conformational switching (Fig. 7). The kinetic and fluorescence data support a model by which the interface-mimicking peptide stabilizes an alternate conformation with reduced enzyme activity and weakened ligand binding. The data also suggest that the inactive conformation induced by the peptide is distinct from the inactive conformation induced by the presence of phosphate. By perturbing domain–domain interactions in the enzyme, this conformational change also reduces DHFR activity.

Novel small molecule inhibitors that bind the apo-state of the human TS active site have recently been discovered.³⁵ These molecules alter the conformation of the active site loop containing the catalytic cysteine, thereby trapping the enzyme in the inactive state.³⁵ The inhibitors display *in vivo* potency against cisplatin-resistant cancer cells, illustrating the potential of the TS inactive state as a therapeutic target.³⁵ Other groups have demonstrated selective inhibition of ThyX, from the flavin-dependent family of TS, by a molecule that binds at the interface between the individual monomers.³⁶ There is, therefore, a potential to take advantage of conformational switching in the design of both active site and allosteric inhibitors of *T. gondii* TS–DHFR.

The lack of an effect of the C21 peptide on the fluorescence signal of *T. gondii* TS–DHFR in the presence of dUMP may explain why the inhibitory effect of the peptides was selective for the apo-enzyme state. The crystal structure of the apo-TS–DHFR from *Babesia bovis*, similar to the TS–DHFR from *T. gondii* in domain organization, showed that the apo form of the enzyme had increased disorder in both the TS and DHFR domains as compared to the dUMP-bound structure.⁹ Peptides from human TS were also dependent on the apo-enzyme state.²³ This further validates the role dUMP plays in transitioning the TS enzyme into a stable, active conformation. We demonstrated the importance of conserved Trp476 for TS catalysis in *T. gondii* TS–DHFR, which had a striking impact on activity despite being a nonactive site residue. Because this residue is located near the interface region and directly upstream of the catalytic cysteine, our data indicate that Trp476 could play a role in proper TS–TS dimerization or proper orientation of the active site. The data suggest that the inhibitory mechanism of the C21 interface peptide involves displacement of the active site loop containing Trp476 and the catalytic cysteine.

The ability of the peptides to inhibit both the TS and DHFR active sites provides further evidence for domain–domain communication in *T. gondii* TS–DHFR. To this end, the mutants RDN, HFRDN, and F319A were constructed. These mutations allowed us to dissect the interactions important for enzyme activity. The RDN mutation's effect on the K_m of

dUMP was similar to those found in crossover helix mutants, but it did not significantly impact DHFR efficiency.²⁵ The F319A single mutant significantly affected DHFR activity, while the HFRDN mutant greatly impacted the enzyme efficiency of both the TS and DHFR active sites and also prevented efficient DHFR inhibition by the peptides. The results show that the interactions involving the mutated residues play a role in stabilizing the enzyme, and mutations perturbing these interactions impact the TS and DHFR catalysis. These mutants also offer clues to explain the molecular mechanism that underlies the crossover helix modulating the activity of the downstream TS domain.²⁵ The data confirm that the TS junctional region of the linker in particular plays a significant role in the mechanism by which the peptides alter the DHFR activity by binding at the TS interface. This suggests that interdomain interactions mediated by the TS junctional region are an important characteristic of the class of bifunctional TS–DHFRs containing a linker.

The observation that interface peptides from human TS inhibit cell growth and intracellular TS activity²³ demonstrates their potential relevance in developing novel therapeutics. Taking advantage of the unique TS–TS interface and molecular motions of the TS dimer in *T. gondii* could allow for the design of selective small molecule, allosteric inhibitors that bind in the same manner as the interface peptides. Taken together, our results suggest that the inactive state of the enzyme, particularly at the TS–TS interface could be a target for the design of small molecules to treat parasitic infections.

Materials and Methods

Chemicals

All buffers and other reagents employed were of the highest commercial purity. 7,8- H_2F was chemically prepared by the reduction of folic acid (Sigma-Aldrich, St. Louis, MO) with sodium hydrosulfite.³⁷ The concentration of H_2F was determined by using a molar extinction coefficient of $28,000\text{ M}^{-1}\text{ cm}^{-1}$. Tritium-labeled and unlabeled $\text{CH}_2\text{H}_4\text{F}$ were synthesized using tritiated ($[3',5',7,9\text{-}^3\text{H}]$) or unlabeled folic acid, respectively, as starting material (Movarek Biochemicals, Brea, CA and Sigma-Aldrich, respectively).^{7,38} Enzymatic conversion of H_2F to (6R,S)-5,6,7,8- H_4F was followed by condensation with formaldehyde to form $\text{CH}_2\text{H}_4\text{F}$. The concentration of $\text{CH}_2\text{H}_4\text{F}$ was determined using a molar extinction coefficient of $32,000\text{ M}^{-1}\text{ cm}^{-1}$. NADPH was purchased from Sigma and dUMP from MP Biomedicals (Santa Ana, CA).

Purification of TS–DHFR

T. gondii TS–DHFR was prepared from *E. coli* BL21 cells (Invitrogen, Carlsbad, CA) freshly transformed

with PET15b plasmid containing the coding sequence for *T. gondii* TS–DHFR. The *T. gondii* TS–DHFR plasmid was a generous gift from Dr. David S. Roos. For RDN and HFRDN mutants, site-directed mutagenesis was performed using a Quik-Change mutagenesis kit (Stratagene, La Jolla, CA). For the crossover helix deletion mutant, 5'-phosphorylated primers (IDT, Coralville, IA) flanking the region to be deleted were used in PCR; the resulting product was then ligated together to delete the desired portion.

The protein was purified using an affinity column of methotrexate bound to agarose (Sigma-Aldrich), as described previously.⁷ A PD-10 column (Amersham Biosciences, Amersham, UK) was used to remove residual H_2F after purification. The concentration of purified *T. gondii* TS–DHFR was determined by spectrophotometrically at 280 nm, using an extinction coefficient of $71,850\text{ M}^{-1}\text{ cm}^{-1}$. The enzyme was flash frozen in storage buffer 25 mM Tris 7.3, 10 mM DTT, and 10% glycerol and stored in -80°C . Structural figures were generated using the program Pymol.³⁹ Sequence alignments were generated using the COBALT alignment software.⁴⁰

The TS–DHFR from *L. major* was purified using similar methods as described previously.⁴¹ Human TS and DHFR were purified using nickel affinity and methotrexate affinity chromatography, respectively, as previously described.^{42,43} The plasmids containing human TS and DHFR were a generous gift from Dr. Frank Maley.

Peptides

The C21 peptide (ChinaPeptides, Shanghai, China) and its fragments (GenScript, Piscataway, NJ) were synthesized to >95% purity as determined by HPLC and mass spectrometry. The C21 peptide was dissolved in dimethylformamide (DMF) while the fragments were solubilized in 1X enzyme buffer (50 mM Tris pH 7.8, 25 mM MgCl_2 , 1 mM EDTA, 10 mM DTT). Experiments with the C21 peptide were performed ensuring a consistent final DMF concentration of 2.5% for all samples.

Steady state kinetic experiments

Experiments in this article were performed in duplicate or triplicate. Steady-state experiments were performed in 1X enzyme buffer. Steady-state peptide inhibition assays were performed using 200 μM $\text{CH}_2\text{H}_4\text{F}$ and 100 μM dUMP for the TS reaction and 100 μM of both H_2F and NADPH for the DHFR reaction. Enzyme either in the apo-state or premixed with dUMP was preincubated with peptide before being mixed with NADPH/ H_2F (DHFR) or $\text{CH}_2\text{H}_4\text{F}$ /dUMP (TS). The DHFR activity was measured by a decrease in absorbance at 340 nm as NADPH and H_2F were converted to NADP^+ and H_4F ($\Delta\epsilon = 10\text{ mM}^{-1}\text{ cm}^{-1}$). The TS activity was measured by an

increase in absorbance at 340 nm as dUMP and CH₂H₄F were converted to dTMP and H₂F ($\Delta\epsilon = 6.4 \text{ mM}^{-1}\text{cm}^{-1}$). Mechanism of inhibition studies were performed by keeping the concentrations of peptide and H₂F (DHFR) or CH₂H₄F (TS) constant and varying the concentration of NADPH (DHFR) or dUMP (TS). The IC₅₀'s and rate constants for steady state experiments were estimated by fitting the data to a Michaelis–Menten hyperbolic curve ($v = V_{\text{max}}[S]/(K_m + [S])$, where v is the reaction rate and $[S]$ the concentration of substrate) using the curve-fitting program, Kaleidagraph (version 4.03, Synergy Software). For the determination of the K_m and k_{cat} of the mutant enzymes, the concentration of either CH₂H₄F (TS) or NADPH (DHFR) were held constant (200 and 100 μM , respectively) while the concentration of dUMP (TS) or H₂F (DHFR) were varied. Enzyme efficiencies were calculated using the equation $\text{Efficiency} = k_{\text{cat}}/K_m$.

Stopped-flow kinetic experiments

Stopped-flow experiments were performed using a Kintek SF-2001 apparatus (Kintek Instruments, Austin, TX).⁷ To determine rate of the DHFR reaction, coenzyme fluorescence resonance energy transfer were carried out with 290 nm excitation and an output filter at 450 nm. Concentrations of enzyme and substrates described are those after mixing. To find the single-turnover rate (k_{chem}) of the DHFR reaction, enzyme (50 μM in 2 $\times$ enzyme buffer: 100 mM Tris pH 7.8, 50 mM MgCl₂, 2 mM EDTA, 2 mM DTT) was incubated with NADPH (250 μM) and then mixed with H₂F (25 μM), and fluorescence was measured. Changes in fluorescence upon mixing were monitored and the resultant data were fit to a single exponential curve, $\text{Fluorescence} = Ae^{(-k_{\text{chem}} \cdot \text{time})} + C$, where A is the amplitude, C is the final value of fluorescence, and k_{chem} is the rate constant.

Rapid chemical quench

The bifunctional TS–DHFR reaction was measured via using a Kintek RFQ-3 Rapid Chemical Quench Apparatus (Kintek Instruments, Austin, TX). The reactions were initiated by mixing 15 μL enzyme solution (enzyme + 2 $\times$ enzyme buffer) with 15 μL radiolabeled CH₂H₄F. The reactions were terminated by quenching with 89 μL of a solution of 0.78 N KOH, 10% sodium ascorbate, and 200 mM 2-mercaptoethanol. The TS–DHFR single-turnover bifunctional reaction was monitored by addition of [³H]-CH₂H₄F (10 μM) to enzyme (50 μM), dUMP (500 μM), and NADPH (500 μM).

High-performance liquid chromatography analysis

Tritiated products of the rapid chemical quench experiments were quantified by HPLC in combina-

tion with a radioactivity flow detector (PerkinElmer, Waltham, MA) as described previously.^{7,19} The HPLC separation was performed using a BDS-Hypersil C18 reverse phase column (Thermo Fisher, Waltham, MA) with a flow rate of 1 ml/min. An isocratic separation using a solvent system of 10% methanol in 200 mM triethylammonium bicarbonate at pH 8.0 was used. The elution times were as follows: H₄F, 9 min; H₂F, 18 min; CH₂H₄F, 20 min. The resultant data were fit to a single exponential curve.

Fluorescence studies

Fluorescence measurements were taken using a QuantaMaster 40 Fluorimeter (Photon Technology International, Birmingham, NJ) and the program FeliXTM. All experiments were performed in 1 $\times$ enzyme buffer at 25°C with 4 μM of enzyme in a 2 mL cuvette. Excitation was set to 295 nm and emission scanning from 305 to 450 nm at a rate of 3 nm/s⁻¹. Ligands were added so as not to exceed 5% of the total volume. No contribution to fluorescence was detected from the enzyme buffer, potassium phosphate, dUMP, DMF, or C21 peptide. Relative fluorescence was calculated by dividing each time point over the peak of fluorescence for the unliganded enzyme (approximately 344 nm).

Electronic Supplementary Material

The file “SupplementalMaterial.pdf” contains electronic supplementary material consisting of five figures with figure legends. These figures show mode of inhibition kinetic analysis, K_d determination from fluorescence data, the effect of the W476A mutant, and additional experiments testing the effect of peptides against the HFRDN mutant.

Acknowledgments

This work is supported by the NIH Grant AI083146 (to K.S.A.) and NIH Grant 5 T32 GM067543 (to M.J.L.). We thank Dr. Ryan Pelto and Dr. Vidya Kumar for their help in purifying dihydrofolate, CH₂H₄F, human TS, and human DHFR.

References

1. Fletcher S, Hamilton AD (2006) Targeting protein-protein interactions by rational design: mimicry of protein surfaces. *J R Soc Interface* 3:215–233.
2. Loregian A, Palu G (2005) Disruption of protein-protein interactions: towards new targets for chemotherapy. *J Cell Physiol* 204:750–762.
3. Hardy LW, Finer-Moore JS, Montfort WR, Jones MO, Santi DV, Stroud RM (1987) Atomic structure of thymidylate synthase: target for rational drug design. *Science* 235:448–455.
4. Schiffer CA, Clifton IJ, Davisson VJ, Santi DV, Stroud RM (1995) Crystal structure of human thymidylate synthase: a structural mechanism for guiding substrates into the active site. *Biochemistry* 34:16279–16287.

5. Anderson AC, O'Neil RH, DeLano WL, Stroud RM (1999) The structural mechanism for half-the-sites reactivity in an enzyme, thymidylate synthase, involves a relay of changes between subunits. *Biochemistry* 38:13829–13836.
6. Chen CH, Davis RA, Maley F (1996) Thermodynamic stabilization of nucleotide binding to thymidylate synthase by a potent benzoquinazoline folate analogue inhibitor. *Biochemistry* 35:8786–8793.
7. Johnson EF, Hinz W, Atreya CE, Maley F, Anderson KS (2002) Mechanistic characterization of *Toxoplasma gondii* thymidylate synthase (TS-DHFR)-dihydrofolate reductase. Evidence for a TS intermediate and TS half-sites reactivity. *J Biol Chem* 277:43126–43136.
8. Perry KM, Pookanjanatavip M, Zhao J, Santi DV, Stroud RM (1992) Reversible dissociation and unfolding of the dimeric protein thymidylate synthase. *Protein Sci* 1:796–800.
9. Begley DW, Edwards TE, Raymond AC, Smith ER, Hartley RC, Abendroth J, Sankaran B, Lorimer DD, Myler PJ, Staker BL, Stewart LJ (2011) Inhibitor-bound complexes of dihydrofolate reductase-thymidylate synthase from *Babesia bovis*. *Acta Cryst F* 67:1070–1077.
10. Knighton DR, Kan CC, Howland E, Janson CA, Hostomska Z, Welsh KM, Matthews DA (1994) Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. *Nat Struct Biol* 1:186–194.
11. O'Neil RH, Lilien RH, Donald BR, Stroud RM, Anderson AC (2003) Phylogenetic classification of protozoa based on the structure of the linker domain in the bifunctional enzyme, dihydrofolate reductase-thymidylate synthase. *J Biol Chem* 278:52980–52987.
12. Senkovich O, Schormann N, Chattopadhyay D (1993) Structures of dihydrofolate reductase-thymidylate synthase of *Trypanosoma cruzi* in the folate-free state and in complex with two antifolate drugs, trimetrexate and methotrexate. *Acta Cryst D* 65:704–716.
13. Yuvaniyama J, Chitnumsub P, Kamchonwongpaisan S, Vanichthanankul J, Sirawaraporn W, Taylor P, Walkinshaw MD, Yuthavong Y (2003) Insights into antifolate resistance from malarial DHFR-TS structures. *Nat Struct Biol* 10:357–365.
14. Fierke CA, Johnson KA, Benkovic SJ (1987) Construction and evaluation of the kinetic scheme associated with dihydrofolate reductase from *Escherichia coli*. *Biochemistry* 26:4085–4092.
15. Belperron AA, Fox BA, O'Neil RH, Peaslee KA, Horii T, Anderson AC, Bzik DJ (2004) *Toxoplasma gondii*: generation of novel truncation mutations in the linker domain of dihydrofolate reductase-thymidylate synthase. *Exp Parasitol* 106:179–182.
16. Gatton ML, Martin LB, Cheng Q (2004) Evolution of resistance to sulfadoxine-pyrimethamine in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 48:2116–2123.
17. Kaufman HE, Geisler PH (1960) The hematologic toxicity of pyrimethamine (daraprim) in man. *Arch Ophthalmol* 64:140–146.
18. Dasgupta T, Anderson KS (2008) Probing the role of parasite-specific, distant structural regions on communication and catalysis in the bifunctional thymidylate synthase-dihydrofolate reductase from *Plasmodium falciparum*. *Biochemistry* 47:1336–1345.
19. Martucci WE, Udier-Blagovic M, Atreya C, Babatunde O, Vargo MA, Jorgensen WL, Anderson KS (2009) Novel non-active site inhibitor of *Cryptosporidium hominis* TS-DHFR identified by a virtual screen. *Bioorg Med Chem Lett* 19:418–423.
20. Trujillo M, Donald RG, Roos DS, Greene PJ, Santi DV (1996) Heterologous expression and characterization of the bifunctional dihydrofolate reductase-thymidylate synthase enzyme of *Toxoplasma gondii*. *Biochemistry* 35:6366–6374.
21. Vargo MA, Martucci WE, Anderson KS (2009) Disruption of the crossover helix impairs dihydrofolate reductase activity in the bifunctional enzyme TS-DHFR from *Cryptosporidium hominis*. *Biochem J* 417:757–764.
22. Roos DS (1993) Primary structure of the dihydrofolate reductase-thymidylate synthase gene from *Toxoplasma gondii*. *J Biol Chem* 268:6269–6280.
23. Cardinale D, Guaitoli G, Tondi D, Luciani R, Henrich S, Salo-Ahen OM, Ferrari S, Marverti G, Guerrieri D, Ligabue A, Frassinetti C, Pozzi C, Mangani S, Fessas D, Guerrini R, Ponterini G, Wade RC, Costi MP (2011) Protein-protein interface-binding peptides inhibit the cancer therapy target human thymidylate synthase. *Proc Natl Acad Sci U S A* 108:E542–E549.
24. Prasanna V, Bhattacharjya S, Balaram P (1998) Synthetic interface peptides as inactivators of multimeric enzymes: inhibitory and conformational properties of three fragments from *Lactobacillus casei* thymidylate synthase. *Biochemistry* 37:6883–6893.
25. Sharma H, Landau MJ, Vargo MA, Spasov KA, Anderson KS (2013) First three-dimensional structure of *Toxoplasma gondii* thymidylate synthase-dihydrofolate reductase: insights for catalysis, interdomain interactions, and substrate channeling. *Biochemistry*, in press.
26. Gibson LM, Celeste LR, Lovelace LL, Lebioda L (2011) Structures of human thymidylate synthase R163K with dUMP, FdUMP and glutathione show asymmetric ligand binding. *Acta Cryst D* 67:60–66.
27. Gibson LM, Lovelace LL, Lebioda L (2008) The R163K mutant of human thymidylate synthase is stabilized in an active conformation: structural asymmetry and reactivity of cysteine 195. *Biochemistry* 47:4636–4643.
28. Lovelace LL, Johnson SR, Gibson LM, Bell BJ, Berger SH, Lebioda L (2009) Variants of human thymidylate synthase with loop 181–197 stabilized in the inactive conformation. *Protein Sci* 18:1628–1636.
29. Luo B, Repalli J, Yousef AM, Johnson SR, Lebioda L, Berger SH (2011) Human thymidylate synthase with loop 181–197 stabilized in an inactive conformation: ligand interactions, phosphorylation, and inhibition profiles. *Protein Sci* 20:87–94.
30. Berger SH, Berger FG, Lebioda L (2004) Effects of ligand binding and conformational switching on intracellular stability of human thymidylate synthase. *Biochim Biophys Acta* 1696:15–22.
31. Felder T, Dunlap RB, Dix D, Spencer T (2002) Differences in natural ligand and fluoropyrimidine binding to human thymidylate synthase identified by transient-state spectroscopic and continuous variation methods. *Biochim Biophys Acta* 1597:149–156.
32. Phan J, Steadman DJ, Koli S, Ding WC, Minor W, Dunlap RB, Berger SH, Lebioda L (2001) Structure of human thymidylate synthase suggests advantages of chemotherapy with noncompetitive inhibitors. *J Biol Chem* 276:14170–14177.
33. Lovelace LL, Gibson LM, Lebioda L (2007) Cooperative inhibition of human thymidylate synthase by mixtures of active site binding and allosteric inhibitors. *Biochemistry* 46:2823–2830.
34. Luo B, Johnson SR, Lebioda L, Berger SH (2011) Evolution of metamorphism in thymidylate synthases within the primate lineages. *J Mol Evol* 72:306–314.
35. Carosati E, Tochowicz A, Marverti G, Guaitoli G, Benedetti P, Ferrari S, Stroud RM, Finer-Moore J,

- Luciani R, Farina D, Cruciani G, Costi MP (2012) Inhibitor of ovarian cancer cells growth by virtual screening: a new thiazole derivative targeting human thymidylate synthase. *J Med Chem* 55:10272–10276.
36. Basta T, Boum Y, Briffotiaux J, Becker HF, Lamarre-Jouenne I, Lambry JC, Skouloubris S, Liebl U, Graille M, van Tilbeurgh H, Myllykallio H (2012) Mechanistic and structural basis for inhibition of thymidylate synthase ThyX. *Open Biol* 2:120120.
 37. Blakley RL (1960) Crystalline dihydropteroylglutamic acid. *Nature* 188:231–232.
 38. Doan LT, Martucci WE, Vargo MA, Atreya CE, Anderson KS (2007) Nonconserved residues Ala287 and Ser290 of the *Cryptosporidium hominis* thymidylate synthase domain facilitate its rapid rate of catalysis. *Biochemistry* 46:8379–8391.
 39. DeLano WL (2002) The PyMol Molecular Graphics System. Palo Alto, CA: DeLano Scientific.
 40. Papadopoulos JS, Agarwala R (2007) COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics* 23:1073–1079.
 41. Atreya CE, Johnson EF, Williamson J, Chang SY, Liang PH, Anderson KS (2003) Probing electrostatic channeling in protozoal bifunctional thymidylate synthase-dihydrofolate reductase using site-directed mutagenesis. *J Biol Chem* 278:28901–28911.
 42. Pedersen-Lane J, Maley GF, Chu E, Maley F (1997) High-level expression of human thymidylate synthase. *Protein Expr Purif* 10:256–262.
 43. Prendergast NJ, Delcamp TJ, Smith PL, Freisheim JH (1998) Expression and site-directed mutagenesis of human dihydrofolate reductase. *Biochemistry* 27:3664–3671.