ACS Medicinal Chemistry Letters

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Discovery of Potent and Selective Inhibitors of *Toxoplasma gondii* Thymidylate Synthase for Opportunistic Infections

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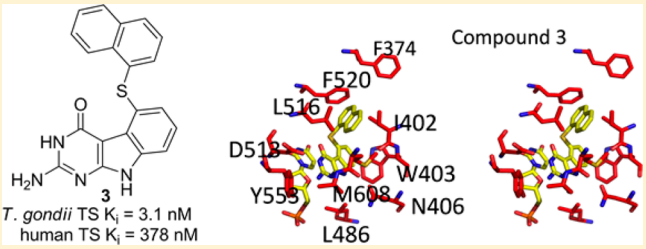
Supporting Information

ABSTRACT: Infection by the parasite *Toxoplasma gondii* (tg) can lead to toxoplasmosis in immunocompromised patients such as organ transplant, cancer, and HIV/AIDS patients. The bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR) enzyme is crucial for nucleotide synthesis in *T. gondii* and represents a potential target to combat *T. gondii* infection. While species selectivity with drugs has been attained for DHFR, TS is much more conserved across species, and specificity is significantly more challenging. We discovered novel substituted-9*H*-pyrimido[4,5-*b*]indoles **1**−**3** with single-digit nanomolar K_i for tgTS, two of which, **2** and **3**, are 28- and 122-fold selective over human TS (hTS). The synthesis of these compounds, and their structures in complex with tgTS-DHFR are presented along with binding measurements and cell culture data. These results show, for the very first time, that, in spite of the high degree of conservation of active site residues between hTS and the parasite TS, specificity has been accomplished via novel structures and provides a new target (TS) for selective drug development against parasitic infections.



KEYWORDS: *Opportunistic infection, Toxoplasma gondii, crystal structure, thymidylate-synthase inhibitors, active-site inhibitors*

According to the World Health Organization, the parasite *Toxoplasma gondii* (*T. gondii*, tg) has infected up to a third of the world's population.[1] Though the advent of antiretroviral therapy (ART) has decreased the incidence of *T. gondii* infection in AIDS patients, the infections still arise because of noncompliance with therapy, discontinuation of therapy because of toxicities with current treatments, and the emergence of drug-resistance strains. Late diagnosis of HIV and the rise of untreated HIV in developing countries also contributes to the persistence of *T. gondii* infections as a clinical issue.[2] *T. gondii* has been defined by the Centers for Disease Control and Prevention as an AIDS defining infection and a major cause of morbidity and indeed mortality in HIV patients as recently as 2009[3] and 2013.[4] Toxoplasma encephalitis (TE) is the principal opportunistic infection (OI) caused by *T. gondii* in AIDS patients. In addition to AIDS, other immune-compromised patients such as those with organ transplants and those undergoing cancer chemotherapy are also highly susceptible to *T. gondii* infections.

Sulfadiazine (SDZ) with pyrimethamine (PM) (Figure 1) is the principal drug combination for *T. gondii* infection along with folinic acid for rescue of host bone marrow toxicity from PM.[2] Alternate first line therapy with sulfamethoxazole (SMX) and trimethoprim (TMP) (Figure 1) is also used.[2] Problems in chemoprophylaxis, treatment failures, high rates of adverse drug reactions, and drug intolerance to these very similar first-line agents, particularly PM and the sulfonamide component,[5] indicate an urgent need for new agents. The most clinically useful combinations, PM/SDZ (with folinic acid rescue) or TMP/SMX, act synergistically.[6] The sulfonamide component inhibits dihydropteroate synthase (DHPS), which catalyzes an early step in the synthesis of folic acid. DHPS catalyzes the synthesis of de novo folate biosynthesis, a pathway absent in the human host. PM and TMP inhibit dihydrofolate reductase (DHFR). DHFR catalyzes the synthesis of tetrahydrofolate (THF) from folic acid as well as dihydrofolate (DHF). Thus,

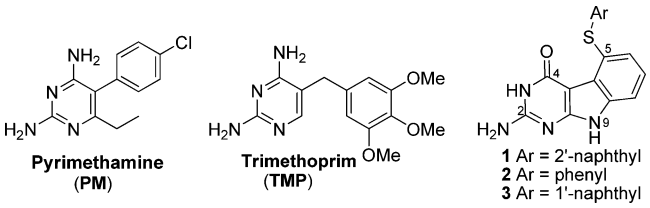


Figure 1. DHFR inhibitors (PM and TMP) in the clinic for *T. gondii* infections and compounds **1**−**3**.

Received: May 29, 2013
Accepted: October 4, 2013
Published: October 4, 2013

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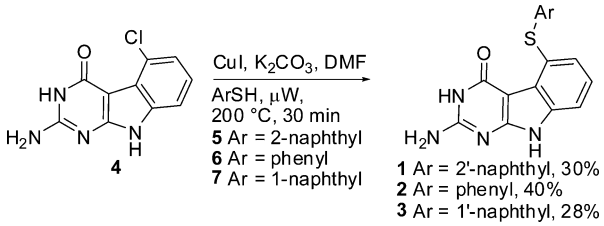
the most successful drugs for *T. gondii* inhibit folate biosynthesis and metabolism at two separate steps to afford inhibition of purine and pyrimidine synthesis. Since Toxoplasma cannot salvage thymidine from the host,[7] indirect inhibition of thymidylate synthase (TS) via DHFR inhibition is possible, but the direct inhibition of TS, the enzyme that catalyzes the conversion of dUMP to dTMP for DNA synthesis would be most logical. However, no selective inhibitors of *T. gondii* TS (tgTS) are known, up to now, that do not also potently inhibit human TS (hTS) as well.[8] Unlike DHFR, where the human and parasite enzymes are somewhat divergent in amino acid sequence and structure allowing for selective inhibition of the parasite DHFR by 100-fold or greater, such divergence is much less in TS from parasites and humans.[9] There is significant homology between the active site residues of tgTS and hTS. The significance of selective inhibition of tgTS is that this would inhibit the replication of *T. gondii* cells since *T. gondii*, unlike human, lacks salvage of thymidine, and thus, TS function in *T. gondii* is obligatory for survival.[10] Such selective inhibitors could be used alone or in combination with PM, TMP, SMX, and other sulfonamides.

On the basis of the potent bicyclic hTS inhibitor nolatrexed reported by Webber et al.,[11] we designed tricyclic compounds **1**−**3** using as a novel scaffold for hTS inhibition and as potential anticancer agents. Compounds **1**−**3** (Figure 1) contain a tricyclic pyrimido[4,5-*b*]indole scaffold connected to a phenyl or a naphthalene moiety via a sulfur bridge. Compounds **1**−**3** were synthesized using Scheme 1. Compound **4** was prepared by a method developed by Gangjee et al.[12] Ullmann coupling[13] of **4** with aryl thiols **5**−**7** in a microwave apparatus (Initiator from Biotage) in the presence of potassium carbonate provided target compounds **1**−**3**, respectively, in yields of 28−40%.

Scheme 1. Synthesis of 1−3



Inhibition assays were performed on isolated *T. gondii* TS-DHFR and hTS. Compounds **1**−**3** exhibited single-digit nanomolar K_i values (Table 1). Surprisingly, compounds **2** and **3** showed unprecedented selectivity for tgTS over hTS of 28- and 122-fold, respectively.

To determine the structural basis for the observed activity and selectivity, we determined the crystal structures of inhibitors **2** and **3** with *T. gondii* TS-DHFR (Figure 2). By superimposing the tgTS and hTS (Figure S3, Supporting Information), almost all of the residues in the active site were identical. It was surprising that a remarkable difference of 28- and 122-fold in the K_i values of **2** and **3**, respectively, were observed in spite of almost identical architectures of the active sites of TS of the two organisms. As shown in Figure 2, the crystal structure of the tgTS-DHFR with **2** and **3** revealed extensive base stacking interactions between the inhibitor and the nucleotide dUMP as expected. Using cocrystal structures of **2** and **3** with tgTS-DHFR, we generated ligand interactions using MOE 2011.10.[14] Compound **2** is bound to the active site by aromatic stacking of the pyrimido[4,5-*b*]indole scaffold and the dUMP pyrimidine ring. Additionally, hydrogen bonds are formed between the N9 of **2** and carboxyl of Asn406, the Asp513 carboxylic oxygen with N3, and the 2-NH_2 group. The 2-NH_2 group forms an additional hydrogen bond with the backbone oxygen of Ala609. The scaffold is also stabilized by hydrophobic interactions of the C-ring with Trp403. The phenyl ring is oriented almost at a right angle to the tricyclic scaffold and interacts with Ile402, Leu516, Phe520, and Met608. Compound **3** retains the interactions of the tricyclic scaffold and the hydrogen bonds with Asp513, Ala609, and Asn409. Similar to **2**, the 1′-naphthyl ring of **3** is oriented almost at a right angle to the tricyclic scaffold and interacts with Ile402, Leu516, Phe520, and Met608.

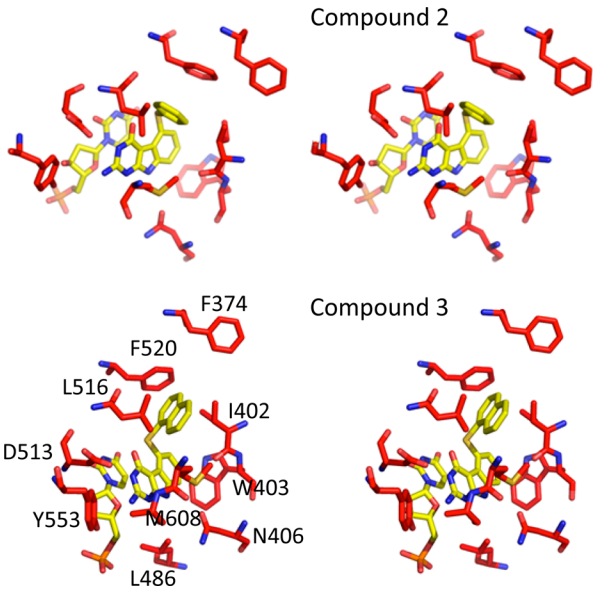


Figure 2. *T. gondii* TS active site with key residues highlighted in red. The inhibitors **2** and **3** and dUMP are colored yellow.

Table 1. Kinetic Evaluation of 1−3 Reveals Species Selectivity[a]

compd	IC_{50} (nM)		K_i (nM)[b]		selectivity (hTS K_i/tgTS K_i)
	tgTS	hTS	tgTS	hTS	
1	21 ± 13	28.8 ± 21	1.8 ± 1.1	3.3 ± 2.4	1.83
2	23 ± 6.6	481 ± 162	2.0 ± 0.6	55.7 ± 18.8	27.9
3	35.8 ± 2.9	3250 ± 184	3.1 ± 0.25	378 ± 21	121.9

[a] *T. gondii* TS-DHFR (25 nM) and human TS (50 nM) were preincubated with 100 µM dUMP and inhibitor. The reaction was initiated with 100 µM methyleneTHF. [b] Because of the concentrations of enzyme required for assay and the limits of detection, for those compounds having K_i values in the lower nanomolar range, the values reported represent an upper limit.

The structure of **3** in the TS active site suggested that the 1′-naphthalene moiety of **3** must be a key determinant of higher selectivity. The position of the compound in the active site orients the 1′-naphthalene moiety close to the C-terminal tail of the *T. gondii* TS domain. In fact, the residues of the C-terminal tail are well ordered only in *T. gondii* and not in the known X-ray crystal structures of human TS. The 1′-naphthalene substituent of **3** also makes hydrophobic interactions with M608 only in tgTS since the C-terminal tail shields the active site from solvent; the tail could be a possible structural determinant that facilitates additional hydrophobic interactions with tgTS conferring selectivity for the parasite (Figure 2).

Compounds **1**−**3** have also been evaluated against *T. gondii* cells in culture (Table 2), and **3**, at 5 μM, was found to be equivalent to PM at 3 μM indicating a significant effect on *T. gondii* in culture comparable to a clinically used agent PM. The culture model requires that the experimental compounds rapidly kill exposed tachyzoites as they are released from cells and/or that the experimental compounds penetrate the cell and the vacuole containing tachyzoites where the compounds may kill the organism or prevent its replication.

Table 2. Toxoplasma Cell Culture Study

condition	*T. gondii* in media at day 4	*T. gondii* in cells at day 4	estimated IC_{50} value (μM)[a]
no drug	3710000	1140000	
1 (5 μM)	2350000	340000	2.6
2 (5 μM)	100000	140000	3.7
3 (5 μM)	<25000	<2500	0.6
PM (3 μM)	<25000	<2500	0.57

[a]Dose−response curves using data from the three concentrations of test compounds gave estimates of IC_{50} values.

We have discovered, for the first time, highly potent (single-digit nM) tgTS inhibitors **1**−**3** with up to a remarkable 122-fold selectivity for tgTS over hTS (K_i in Table 1). This level of selectivity is unprecedented for any parasite with regard to TS. For comparison, TMP selectively inhibits tgDHFR and is used extensively with SMX to treat Toxoplasma infection but has an IC_{50} = 2.7 μM and a selectivity of only 49-fold for tgDHFR over hDHFR.[15] Further PM, a widely used gold standard tgDHFR inhibitor for TE and other *T. gondii* infections, only has a potency of IC_{50} = 3.9 μM and a selectivity of only 5.9 for tgDHFR over mammalian DHFR. In addition, we have also, for the very first time, solved the X-ray crystal structure of the bifunctional tgTS-DHFR enzyme with compounds **2** and **3**.[16] In cell cuture study, compound **3** showed similar potency to PM, a highly effective standard drug used as a control in these studies. We have thus demonstrated with **2** and **3** that structure variation affords increased selectivity while maintaining potency (compared to **1**). Figure S3, Supporting Information, shows dUMP and compound **3** and the superposition of key active site residues (using tg numbering) in both human and tgTS. While all residues are identical between the two species, only M608 is present in the tgTS structure. Future studies will be directed toward solving the structure of compound **3** with human TS to more fully discern possible differences. TS is a completely new and novel target for *T. gondii*. There is no report, to our knowledge, of selective and potent inhibitors of tgTS as anti-OI agents. The ability to provide agents that are selective for tgTS on the basis of structure-based rational design is a major step toward providing novel clinically useful agents with tgTS inhibition.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, compound characterization, biological assays, and X-ray structure determination. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The atomic coordinates of *T.gondii* TS-DHFR/Compound **2** and *T.gondii* TS-DHFR/Compound **3** described here have been deposited in the RCSB Protein Data Bank (www.pdb.org) with accession codes 4KY4 and 4KYA, respectively.

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Funding

This work was supported, in part, by NIH grant CA152316 (to A.G.), the Duquesne University Adrian Van Kaam Chair in Scholarly Excellence (to A.G.), and AI083146 (to K.S.A.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

tg, *T. gondii*; TS, thymidylate synthase; DHFR, dihydrofolate reductase; TE, toxoplasma encephalitis; OI, opportunistic infection; SDZ, sulfadiazine; PM, pyrimethamine; SMX, sulfamethoxazole; TMP, trimethoprim

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