Discovery of potent and selective inhibitors of *Toxoplasma gondii* thymidylate synthase for opportunistic infections.

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SUPPORTING INFORMATION

Synthetic Procedures and Compound Characterization

General Information

Analytical samples were dried in vacuo (0.2 mm Hg) in a CHEM-DRY drying apparatus over P_2O_5 at 80 ^oC. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (^1H NMR) were recorded on Bruker Avance II 400 (400 MHz) and 500 (500 MHz) NMR systems. The chemical shift values are expressed in ppm (parts per million): s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC)

was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230-400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within ±0.4% of the calculated values and indicate >95% purity. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented despite 24-48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. Mass spectrum data were acquired on an Agilent G6220AA TOF LC/MS system using the nano ESI (Agilent chip tube system with infusion chip). All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received.

General procedure for the synthesis of 5-[(4-substitutedphenyl)sulfanyl]-9*H*-pyrimido[4,5-*b*]indole-2,4-diamines 1-3. Compound **4** (50 mg, 0.21 mmol), the appropriate thiol (0.42 mmol), potassium carbonate (120 mg, 0.85 mmol), and copper iodide (162 mg, 0.85 mmol) were added to a 2-5 mL biotage® microwave vial. Around 2 mL DMF was added as solvent and the tube was sealed. The reaction was run in a microwave for 30 minutes at 200 °C (the pressure could be up to 18-20 bar). After cooling to room temperature, the reaction mixture was filtered off and washed with 10 mL of DMF. To the filtrate was added 0.25 g of silica gel and the solvent was removed under reduced pressure to offer a plug, which was transferred on top of a silica gel

column (15 x 100 mm) and eluted with 0%, 2%, and 4% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford the product.

2-Amino-5-(2-naphthylsulfanyl)-3,9-dihydro-4*H*-pyrimido[4,5-*b*]indol-4-one (1). Using the general procedure described above, the reaction of **4** with naphthalene-2-thiol **5** afforded **1** as an off-white solid in 30% yield. TLC R_f 0.47 ($CHCl_3$/MeOH 5:1 with two drops concentrated NH_4OH); mp >250 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 6.54 (br, 2H, NH_2, exch), 6.56 (dd, 1H, J_1 = 7.8 Hz and J_2 = 0.9 Hz, C6-CH), 6.96 (t, 1H, J = 7.8 Hz, C7-CH), 7.1 (dd, 1H, J_1 = 7.8 Hz and J_2 = 0.9 Hz, C8-CH), 7.42 (m, 1H, Ar), 7.52-7.54 (m, 2H, Ar), 7.87-7.98 (m, 4H, Ar), 10.35 (s, 1 H, 3-NH, exch), 11.56 (s, 1 H, 9-NH, exch). Anal. ($C_{20}H_{14}N_4OS \cdot 0.6\ CH_3OH$) C, H, N, S. HRMS (EI): calcd. for $C_{20}H_{14}N_4OS$ *m/z* = 358.0888, found *m/z* = 358.0866.

2-Amino-5-(phenylsulfanyl)-3,9-dihydro-4*H*-pyrimido[4,5-*b*]indol-4-one (2). Using the general procedure described above, the reaction of **4** with benzene thiol **6** afforded **2** as a brown solid in 40% yield. TLC R_f 0.41 ($CHCl_3$/MeOH, 5:1 with two drops concentrated NH_4OH); mp 247 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 6.51 (dd, 1H, J_1 = 7.7 Hz and J_2 = 0.7 Hz, C6-CH), 6.52 (br, 2H, NH_2, exch), 6.96 (t, 1H, J = 7.8 Hz, C7-CH), 7.08 (d, 1H, J_1 = 7.8 Hz and J_2 = 0.7 Hz, C8-CH), 7.31-7.39 (m, 5H, Ar), 10.34 (s, 1H, 3-NH, exch), 11.54 (s, 1H, 9-NH, exch). Anal. ($C_{16}H_{12}N_4OS \cdot 0.23\ CH_3OH$) C, H, N, S.

2-Amino-5-(1-naphthylsulfanyl)-3,9-dihydro-4*H*-pyrimido[4,5-*b*]indol-4-one (3). Using the general procedure described above, the reaction of **4** with naphthalene-1-thiol **7** afforded **3** as a white solid in 28% yield. TLC R_f 0.46 ($CHCl_3$/MeOH 5:1 with two drops concentrated NH_4OH); mp >250 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 6.05 (dd, 1H, J_1 = 7.8 Hz and J_2 = 0.5 Hz, C6-CH), 6.54 (br, 2H, NH_2, exch), 6.76 (t, 1H, J = 7.8 Hz, C7-CH), 6.98 (d, 1H, J_1 = 7.8 Hz and J_2 = 0.5 Hz, C8-CH), 7.48-7.57 (m, 3H, Ar), 7.73-7.75 (m, 1H, Ar), 7.99-8.02 (m, 2H, Ar), 8.19-8.21

(m, 1H, Ar), 10.40 (s, 1 H, 3-NH, exch), 11.53 (s, 1 H, 9-NH, exch). Anal. ($C_{20}H_{14}N_4OS \cdot 0.34\ CH_3OH$) C, H, N, S.

Expression, Purification, and crystallization of *Toxoplasma gondii* TS-DHFR

Wild-type *Toxoplasma gondii* TS-DHFR was expressed and purified as described previously.[1] The loop truncated form of thymidylate synthase dihydrofolate reductase TS-DHFR enzyme was purified as described before for structure determination. Wildtype TS-DHFR was used for kinetic assays. Briefly, the TS-DHFR was overexpressed in *E. coli* BL21 cells after induction with IPTG. In the final step, the protein was stored in buffer containing 25 mM Tris pH 7.3 and 10 mM DTT. The ligands NADPH, methotrexate, dUMP and the inhibitor were added to a final concentration of 500 µM. Crystallization was achieved at a concentration of 10 mg/mL with a 1:1 ratio of the enzyme and the well solution containing PEG 3350 and Potassium Phosphate. The crystals were cryoprotected in mother liquor containing ethylene glycol and frozen in stepwise transfers into liquid nitrogen. Data were collected at beamlines X25 and X29 at Brookhaven National Laboratory.

Structure Determination

Initial processing of X-ray data was accomplished by using HKL2000.[2] The loop truncated model (PDB accession code 4EIL) was used as the search model for molecular replacement by using PHASER.[3] Refinement of the structure was carried out by REFMAC.[4] The ligands and their topology files were generated by the PRODRG server,[5] and manual adjustments to model

and the ligands were made in COOT.[6] Figures were generated using PYMOL.[7] Refinement statistics are shown in Supplementary Table 1.

IC_{50} determination

Separately, the *T. gondii* TS-DHFR (25 nM) and human TS (50 nM) were incubated with dUMP and inhibitor both at 100 μM, and the reaction was initiated with methylene tetrahydrofolate 100 uM. The change in absorbance was monitored by TECAN plate reader at 340 nM. The data was plotted using KALEIDAGRAPH. The IC_{50} values were then determined, from which K_i was determined.

The nucleotide dUMP and the cofator NADPH was purchased from SIGMA Aldrich. Methylene tetrahydrofolate was synthesized by using previous methods.

***T. gondii* Cell Culture Study**

T. gondii strain PHΔHX was grown in culture on an immortalized human cell line (H-tert; human telomerase reverse transcriptase) both to prepare innoculum and for the experimental drug tests. Freshly lysed parasites were removed from culture, resuspended in sterile PBS (phosphate buffered saline), counted, and diluted in medium to allow addition of 1300 *T. gondii* tachyzoites to each well of 24-well culture plates containing monolayers of H-tert cells. After incubation for 4 h at 37 ºC, each well received either 500 µL of media (DMEM, 1% fetal calf serum), or media including various concentrations (5, 0.5, 0.05 µM) of experimental drug. Wells were evaluated visually and one set of wells was harvested daily for four days. Media (10 µL) from harvested wells was spread over a 1 cm square area on a microscope slide; cells in harvested wells were scraped and resuspended in 100 µL of fresh medium and 10 µL was spread over a 1 cm square area on a microscope slide. After air-drying, each slide was fixed in methanol and stained with Giemsa Plus. Organisms and cells/1000x field were counted as an indication of growth of *T. gondii* in the presence or absence of test compounds or pyrimethamine, which served as the positive control.

By day 2 of growth, *T. gondii* tachyzoites began to be detected in the sampled control cell monolayers. By day 4, untreated monolayers were mostly lysed with numerous tachyzoites in both media samples and cell samples. In the treated wells, no *T. gondii* were detected in medium for pyrimethamine and for compound **3**; in the cell monolayer samples, no or rare organisms

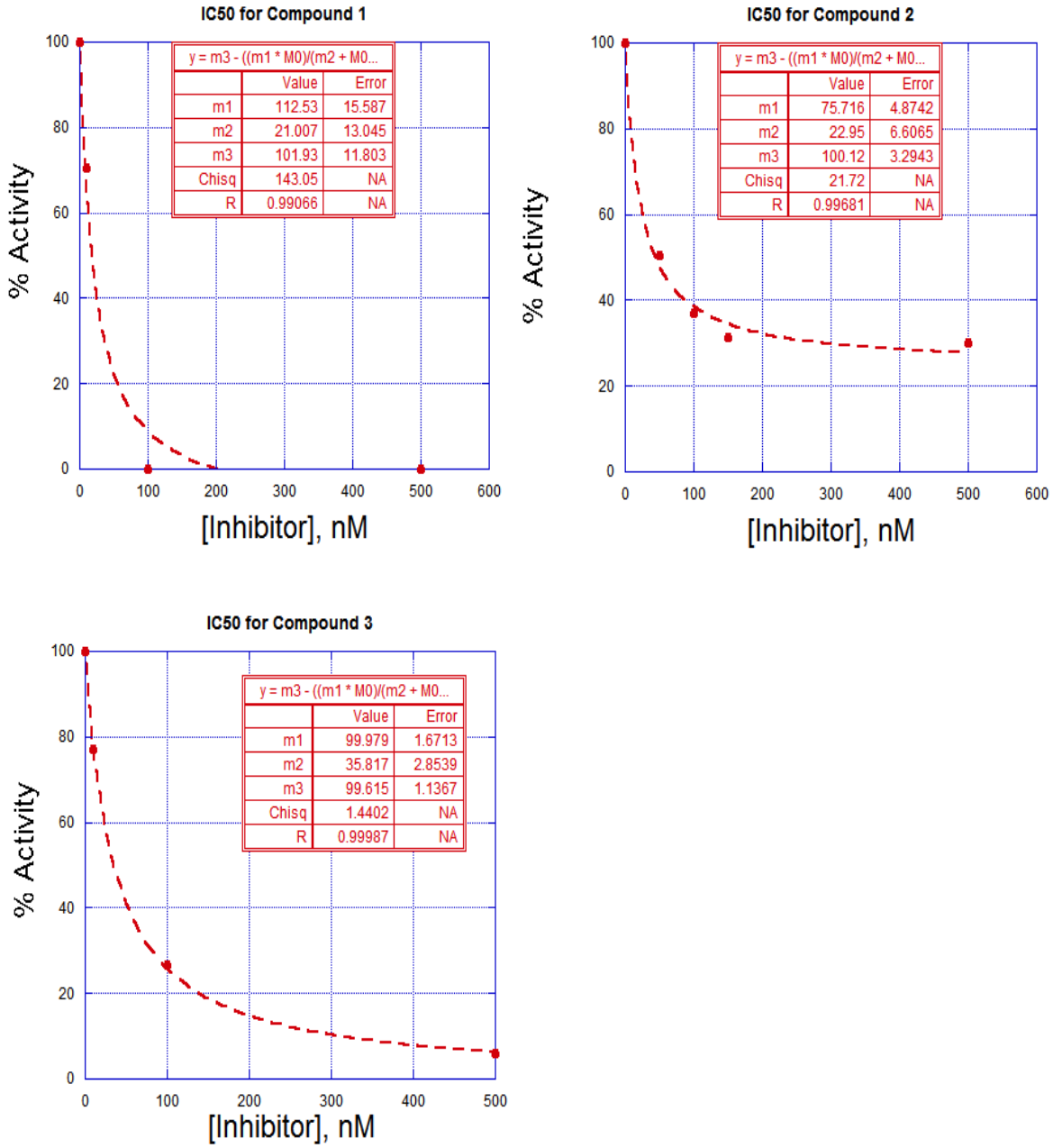
were detected in the cell samples from wells receiving pyrimethamine (3μM) or compound **3** (5μM).

Supplementary Table 1: Crystallographic Statistics for Data Collection and Refinement

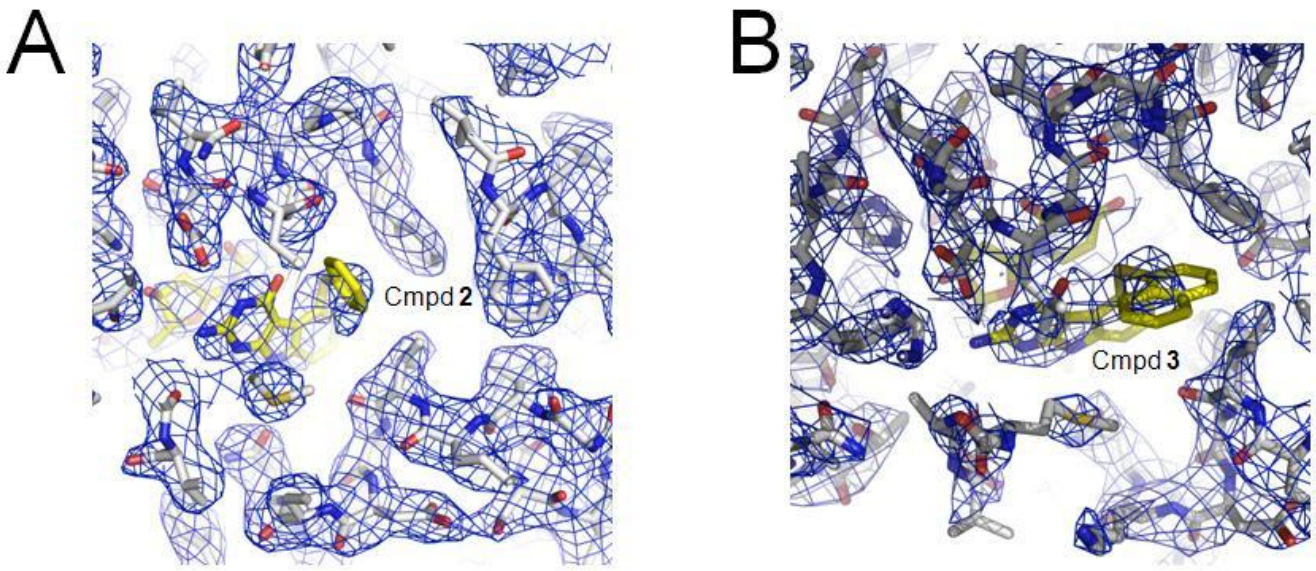
Molecule	Compound **2**/TS-DHFR	Compound **3**/TS-DHFR
Space group	P1	P1
Unit cell dimensions		
a, Å	53.5	53.5
b, Å	145.1	145.1
c, Å	176.2	176.1
Resolution (Å)	48.4-2.8 (2.9-2.8)	48.4-3.3 (3.34-3.26)
Wavelength (Å)	1.1	1.1
Completeness (%)	97.8 (90.3)	98.6 (93.0)
R_{cryst} %	22.0	22.2
R_{free} %	26.8	26.7
I/σ	5.42 (2.15)	10.98 (2.03)
rmsd bond length, Å	0.012	0.011
rmsd bond angle, °	1.636	1.467
Protein statistics from Ramachandran plot		
Residues in favored regions	95.0%	94.8%
Residues in allowed regions	3.3%	4.0%
Outliers	1.7%	1.2%

The values in parentheses are for the highest-resolution shell. R_{merge} is $\Sigma|I_j-\langle I\rangle|/\Sigma I$, where I_j is the intensity of an individual reflection, and $\langle I\rangle$ is the mean intensity for multiple recorded reflections. R_{cryst} is $\Sigma\ |F_o - F_c|/\ \Sigma F_o$, where F_o is an observed amplitude and F_c a calculated amplitude; R_{free} is the same statistic calculated over a subset, 5%, of the data that have not been used for refinement.

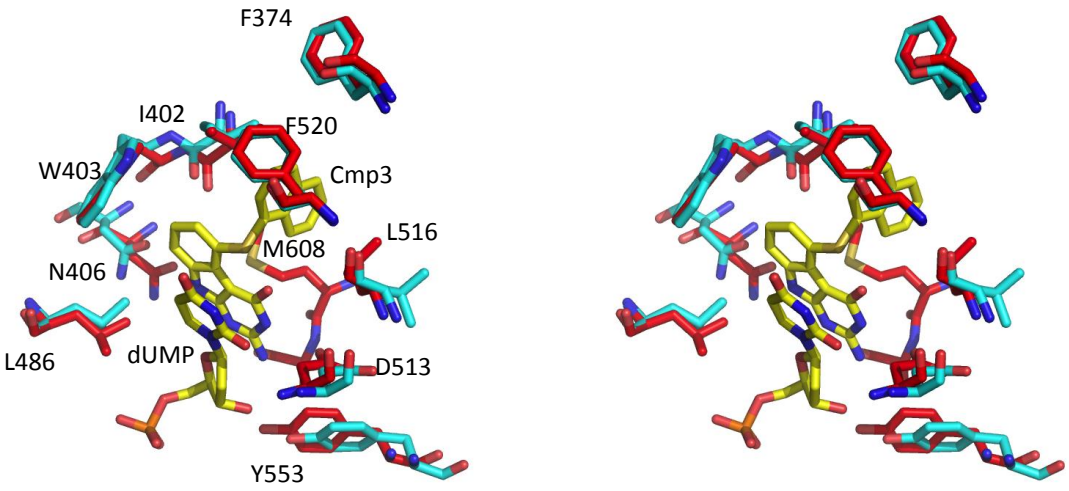
Supplementary Figures



Supplementary Figure 1. IC_{50} determination for compounds **1**, **2**, and **3**.



Supplementary Figure 2. Unbiased electron density of A. compound **2** (yellow) and B. compound **3** (yellow), and the *T. gondii* TS active site (white).



Supplementary Figure 3. TgTS (red) and hTS (cyan) were superimposed. The active site residues interacting with nucleotide and the inhibitor (both yellow) are shown and labeled.

References for Supporting Information

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