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STRUCTURE–ACTIVITY RELATIONSHIP OF LIGANDS OF URACIL PHOSPHORIBOSYLTRANSFERASE FROM *TOXOPLASMA GONDII*

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Abstract—One hundred compounds were evaluated as ligands of *Toxoplasma gondii* uracil phosphoribosyltransferase (UPRTase, EC 2.4.2.9) by examining their ability to inhibit this enzyme *in vitro*. Inhibition was quantified by determining apparent K_i values for those compounds that inhibited *T. gondii* UPRTase by greater than 10% at a concentration of 2 mM. Five compounds (4-thiopyridine, 2-thiopyrimidine, trithiocyanuric acid, 1-deazauracil and 2,4-dithiouracil) bound to the enzyme better than two known substrates for *T. gondii* UPRTase, 5-fluorouracil and emimycin, which have antitoxoplasmal activity (Pfefferkorn ER, *Exp Parasitol* **44**: 26–35, 1978; Pfefferkorn *et al.*, *Exp Parasitol* **69**: 129–139, 1989). In addition, several selected compounds were evaluated as substrates for *T. gondii* UPRTase, and it was found that 2,4-dithiouracil is also a substrate for this enzyme. On the basis of these data, a structure–activity relationship for the binding of ligands to *T. gondii* UPRTase was determined using uracil as a reference compound.

Key words: Toxoplasma gondii; uracil; phosphoribosyltransferase; structure–activity; ligands; inhibitors

Toxoplasma gondii, an obligate intracellular protozoan parasite (for review, see Ref. 1), is one of the many opportunistic infections that afflict individuals suffering from AIDS†. The primary pathology in these patients is toxoplasmic encephalitis, which is reported to occur in 3–40% of AIDS patients [2]. The current standard initial therapy for toxoplasmosis is a combination of pyrimethamine and sulfadiazine, which act synergistically to inhibit folate metabolism [2, 3]. The initial response rate to this therapy is relatively high; however, up to 50% of patients are ultimately treatment failures, primarily due to drug toxicity, which is observed in approximately 60% of patients treated with this regimen [4]. Thus, alternative therapies for the treatment of this disease need to be identified.

Two compounds that have been shown to be effective against *T. gondii* infections in cell culture are the anticancer drug, 5-fluorouracil [5], and the antimicrobial product of a *Streptomyces* species, emimycin (3-hydroxypyrazine-1-oxide) [6]. 5-Fluorouracil is effective (>90% inhibition of parasite replication) at a concentration of 1 μM, while emimycin is effective (>90% inhibition of parasite replication) at a slightly higher concentration (5–10 μM). The initial "activation" of both of these compounds is conversion to their nucleoside 5′-monophosphate forms. This activation occurs in *T. gondii* via the enzyme UPRTase (EC 2.4.2.9) [6, 7], which catalyzes the specific synthesis of uridine 5′-monophosphate from uracil and PRibPP.

UPRTase is a unique target in *T. gondii*, relative to its host, because it is not present in mammalian cells [8]. In addition, UPRTase is a key enzyme in the salvage of pyrimidines in *T. gondii* because all pyrimidine nucleosides and nucleobases must be metabolized through this enzyme [9, 10]. Thus, UPRTase is a good chemotherapeutic target in *T. gondii* because inhibitors of this enzyme may prevent the salvage of pyrimidines in these parasites but not in the host cells. Alternatively, UPRTase could specifically convert ("activate") pyrimidine nucleobase analogs into antitoxoplasmal agents by metabolizing them to their respective nucleoside 5′-monophosphate forms.

Despite its significance as a chemotherapeutic target, very little is known about the binding specificity of *T. gondii* UPRTase. Therefore, in the present study, one hundred compounds were evaluated as ligands of *T. gondii* UPRTase by examining their ability to inhibit this enzyme *in vitro*. Inhibition was quantified by determining appK_i values for those compounds that inhibited *T. gondii* UPRTase by greater than 10% at a concentration of 2 mM. Five compounds (4-thiopyridine, 2-thiopyrimidine, trithiocyanuric acid, 1-deazauracil and 2,4-dithiouracil) bound to the enzyme better than either emimycin or 5-fluorouracil. In addition, several selected compounds were evaluated as substrates for *T. gondii* UPRTase, and it was found that in addition to emimycin and 5-fluorouracil, 2,4-dithiouracil is also a substrate for this enzyme. On

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† Abbreviations: AIDS, acquired immunodeficiency syndrome; appK_i, apparent K_i; PRibPP, 5-phosphoribosyl-1-pyrophosphate; and UPRTase, uracil phosphoribosyltransferase.

the basis of these data, a structure–activity relationship for the binding of ligands to *T. gondii* UPRTase was determined in order to provide the basis for the rational design of additional ligands of this enzyme.

MATERIALS AND METHODS

Materials

Chemicals and supplies. The sources of the compounds screened as inhibitors of UPRTase are indicated in Table 1 by the following abbreviations: ALD, Aldrich Chemical Co., Inc., Milwaukee, WI; AMB, American Bioorganics, North Tonawanda, NY; ERP, Dr. Elmer R. Pfefferkorn, Department of Microbiology, Dartmouth Medical School, Hanover, NH; MHK, Dr. Mahmoud H. el Kouni, Department of Pharmacology, University of Alabama at Birmingham, Birmingham, AL; SCH, Schweizerhall, Inc., Piscataway, NJ; and SIG, Sigma Chemical Co., St. Louis, MO. [2-^{14}C]Uracil (56 mCi/mmol) was obtained from Moravek Biochemicals, Inc., Brea, CA; Scintilene scintillation fluid was from Fisher Scientific, Pittsburgh, PA; silica gel G/UV_{254} Polygram thin-layer chromatography plates were from Brinkmann, Westbury, NY; and Bio-Rad protein assay kits were from Bio-Rad Laboratories, Richmond, CA. All other chemicals and compounds were obtained from either the Sigma Chemical Co. or Fisher Scientific.

Source of T. gondii. Tachyzoites of the RH strain of *T. gondii* were propagated by intraperitoneal passage in female Swiss-Webster mice (Sasco, Inc., Omaha, NE) as previously described [9].

Methods

Preparation of cytosol extracts. Approximately 5×10^8 *T. gondii* were suspended in 1.2 mL of 50 mM Tris–Cl (pH 8.5)/1 mM dithiothreitol and homogenized (on ice) for 30 sec at setting 10, using a Brinkmann Instruments Polytron homogenizer fitted with a PTA 7K1 probe. The homogenate was then centrifuged at approximately 116,000 *g* for 1 hr at 5°, and the supernatant (cytosol extract) was collected.

UPRTase inhibition assays. UPRTase activity was measured by following the formation of [^{14}C]UMP and [^{14}C]uridine (due to phosphohydrolase activity) from [^{14}C]uracil and PRibPP, at the optimal pH (8.5) for the *T. gondii* enzyme (results not shown). The standard reaction mixture contained 50 mM Tris–Cl (pH 8.5), 1 mM dithiothreitol, 10 μM [2-^{14}C]uracil (56 mCi/mmol), 2.5 mM PRibPP, 5 mM $MgCl_2$, 30 μL of cytosol extract (approximately 10 μg of protein), and either 0, 0.25, 0.5, 1 or 2 mM of the compound to be tested, in a final volume of 150 μL. Reactions were started by the addition of [^{14}C]uracil, incubated at 37° for 20 min and terminated by placing the reaction tubes in a boiling water bath for 2 min. Precipitated proteins were removed by centrifugation in a microcentrifuge (approximately 13,000 *g*) for 5 min, and a 15-μL aliquot of the resulting supernatant was mixed with 5 μL of a solution containing 10 mM each of UMP, uridine and uracil. This mixture was then spotted on silica gel thin-layer chromatography plates, which were developed with a mixture of chloroform:methanol:acetic acid (20:4:1). The average R_f values for UMP, uridine and uracil were 0, 0.37 and 0.69, respectively. The substrate and product spots were identified by UV (254 nm) quenching and cut out, and the radioactivity was quantified by liquid scintillation counting in 20 mL of Scintilene using a Packard 460 scintillation counter. All assays were run under conditions in which velocity was linear with respect to time and amount of cytosol extract.

Evaluation of compounds as substrates. Reaction mixtures contained 50 mM Tris–Cl (pH 8.5), 1 mM dithiothreitol, 2.5 mM PRibPP, 5 mM $MgCl_2$, 20 mM NaF, 240 μL of cytosol extract (approximately 80 μg of protein), and 0.1 mM of the compound to be tested, in a final volume of 400 μL. Reactions were started by the addition of cytosol, incubated at 37° for 16 hr, and terminated by adding 23 μL of 70% perchloric acid (final concentration of 4%). The solution was then neutralized by adding 60 μL of 5 N potassium hydroxide and 110 μL of 0.5 M potassium phosphate (pH 7.0). Precipitated proteins and potassium perchlorate were removed by centrifugation in a microcentrifuge (approximately 13,000 *g*) for 5 min. The supernatant was then collected, frozen, and stored at −20°. Samples were thawed by centrifugation and passed through nylon filters (0.45 μm pore size) to remove any particulate matter prior to analysis. The nucleotide contents of the samples were analyzed using a Rainin HPLC system equipped with a Vydac (301-TP104) anion exchange column (25 cm × 4.6 mm). A 200-μL aliquot of each sample was injected onto the column, which was then eluted isocratically with 1 mM potassium phosphate (pH 4.0) for 10 min, followed by a linear gradient (1–500 mM) of potassium phosphate (pH 4.0) for 30 min, at a flow rate of 1 mL/min. Bases and nucleotides were monitored at 254 nm using a Gilson model 112 detector.

Calculations and statistical analysis. Enzyme velocity was calculated by multiplying the fraction of UMP and uridine formed from uracil times the amount of uracil in the assay, and dividing by the incubation time. AppK_i values were estimated from Dixon plots of the data ($1/v$ versus $[I]$) using a computer program that employs the general principles of Cleland [11]. This program was developed by Dr. Sungman Cha (Brown University, Providence, RI) and fitted into IBM BASIC by Dr. Fardos N. M. Naguib (University of Alabama at Birmingham, Birmingham, AL). If a compound is a competitive inhibitor with respect to uracil, appK_i values are related to K_i values by the following equation [12]: $\text{app}K_i = K_i\ (1 + [S]/K_m)$. In the present study, the concentration of uracil (10 μM) was about 4-fold less than its approximate K_m value [9], and the concentration of PRibPP (2.5 mM) was at a saturating concentration for *T. gondii* UPRTase (results not shown). Thus, the appK_i value determined for a competitive inhibitor would be about 1.3-fold higher than the K_i. It should be noted, however, that the type of inhibition (i.e. competitive, noncompetitive, or uncompetitive) produced by the compounds was not determined.

Protein determinations. Protein concentrations were determined by the method of Bradford [13]

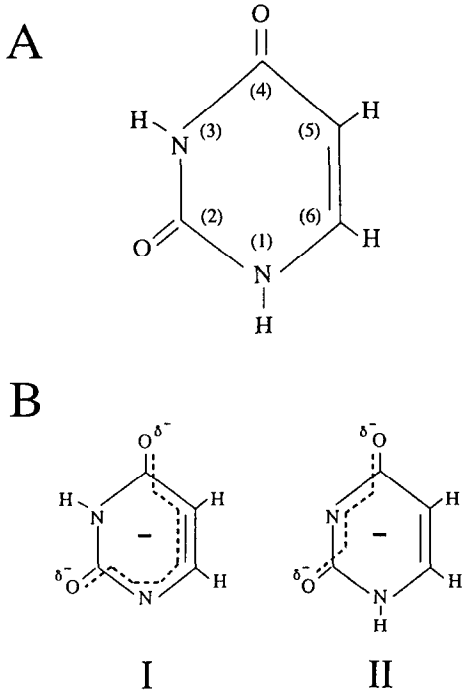


Fig. 1. Tautomerism and ionization of uracil. Panel A shows the diketo tautomeric form of uracil which predominates in solution [14] and the numbering system for the pyrimidine ring. Panel B shows the monoanions of uracil formed by loss of either the *N1* (**I**) or *N3* (**II**) proton [14].

using the Bio-Rad Laboratories protein assay kit and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Determination of appK_i *values*

One hundred compounds were evaluated as ligands of *T. gondii* UPRTase by examining their ability to inhibit this enzyme *in vitro*. Compounds that were screened included uracil analogs with various exocyclic substitutions on the pyrimidine ring (Fig. 1, panel A), as well as several triazine, pyrazine and pyridine analogs. These latter compounds can be considered to be uracil analogs in which endocyclic imine or methylene groups are replaced with endocyclic methylene or imine groups, respectively. AppK_i values were determined for those compounds that inhibited UPRTase by greater than 10% at the highest concentration tested (2 mM). The mean and standard deviation of the appK_i values for these compounds, determined from at least three separate estimations of the appK_i, are shown in Table 1.

Tautomerism and ionization of uracil

Uracil (**1**), which is the natural substrate for *T. gondii* UPRTase, was found to be the best ligand for this enzyme (Table 1). Therefore, a discussion of the tautomerism and ionization of uracil is appropriate. Although uracil can theoretically exist in six possible tautomeric forms, evidence indicates that the diketo form (Fig. 1, panel A) predominates in solution [14]. Ionization of uracil occurs by dissociation of a proton from either the *N1* or *N3* endocyclic nitrogen in approximately a 1:1 ratio [14]. This results in the formation of two different monoanions in which the negative charge is dispersed over the π-electron system of the pyrimidine ring between the 2- and 4-position exocyclic oxygens (Fig. 1, panel B). However, uracil is ionized only 9% under the conditions employed in the present study (Table 2); therefore, it is very likely that *T. gondii* UPRTase preferentially binds the unionized form of uracil rather than the ionized form.

Binding of compounds to T. gondii *UPRTase*

The results shown in Table 1 will be discussed with respect to the effect of substitutions, at the various positions of uracil (Fig. 1, panel A), on binding to *T. gondii* UPRTase. Compounds will be referred to by name and by compound number (bold-type numerals).

1-Position substitutions. An endocyclic imine group at the 1-position is not required for binding, as replacement with a methylene group ("1-deazauracil", **88**) diminished binding by only 4-fold relative to uracil. These results suggest that neither the *N1* nitrogen nor the *N1* proton plays a significant role in binding to the catalytic site of UPRTase. It should be noted, however, that 1-deazauracil is ionized 100% under the conditions employed (Table 2), and ionization occurs at the 4-position exocyclic oxygen rather than the *N3* proton (Fig. 2). Thus, the resulting negative charge on the 4-position oxygen may enhance binding and offset a decrease in binding caused by the absence of the *N1* nitrogen.

Exocyclic substitutions at the 1-position (benzyl, **2**; cyclohexyl, **3**; methyl, **4**; ribose, **5**) abolished binding. The lack of binding of these compounds is probably not due to the absence of an *N1* proton, as this does not appear to affect binding (see above). It is also unlikely that ionization of these compounds affects binding, since their level of ionization is similar to that of uracil (Table 2). One possibility is that the reaction mechanism of *T. gondii* UPRTase is such that PRibPP binds to the enzyme first, followed by uracil. Thus, any exocyclic substituents at the 1-position larger than a hydrogen would preclude binding to UPRTase due to overlap with the PRibPP binding site. This is best exemplified by the fact that uridine (**5**), which is structurally related to two natural ligands of UPRTase (uracil and UMP), does not bind to the enzyme.

2- and 4-Position substitutions. A thio group at either the 2- or 4-position, or a combination of thio and oxo groups at both the 2- and 4-positions, is required for binding. Elimination of one (4-hydroxypyrimidine, **7**; 2-hydroxypyrimidine, **12**) or both (pyrimidine, **44**) of the oxo groups of uracil, substitution of an oxo group with an amino group (2-aminouracil, **6**; 4-aminouracil, **11**), or substitution of both oxo groups with methoxy groups, (2,4-dimethoxypyrimidine, **41**) abolished binding to UPRTase. However, compounds that had a thio group at the 2- or 4-position still retained some binding to the enzyme. For example, the pyrimidine

Table 1. AppK_i values for inhibition of *T. gondii* UPRTase*

	Compound	Source	AppK_i (μM)
Pyrimidine compounds			
1	Uracil	SIG	53 ± 10
	1-Substitutions		
2	1-Benzyluracil	MHK	†
3	1-Cyclohexyluracil	SIG	‡
4	1-Methyluracil	SIG	†
5	1-Ribouracil (uridine)	SIG	‡
	2-Substitutions		
6	2-Aminouracil (isocytosine)	SIG	†
7	4-Hydroxypyrimidine ("2-deoxyuracil")	SIG	‡
8	2-Thiouracil	SIG	4400 ± 1400
	3-Substitutions		
9	3-Butyluracil	SIG	†
10	3-Methyluracil	SIG	†
	4-Substitutions		
11	4-Aminouracil (cytosine)	SIG	†
12	2-Hydroxypyrimidine ("4-deoxyuracil")	SIG	‡
	5-Substitutions		
13	5-Aminouracil	SIG	†
14	5-Benzyluracil	MHK	†
15	5-Bromouracil	SIG	†
16	5-Carbethoxyuracil	SIG	†
17	5-Carboxyuracil (isoorotic acid)	SIG	†
18	5-Chlorouracil	SIG	†
19	5-Diazouracil	SIG	1300 ± 300
20	5-Ethyluracil	SIG	†
21	5-Fluorouracil	SIG	280 ± 100
22	5-Hydroxyuracil (isobarbituric acid)	SIG	‡
23	5-Iodouracil	SIG	†
24	5-Methyluracil (thymine)	SIG	†
25	5-Nitrouracil	SIG	†
26	5-(*n*-Propyl)uracil	SIG	†
27	5-Sulfaminouracil	SIG	†
28	5-Trifluoromethyluracil	SIG	†
	6-Substitutions		
29	6-Aminouracil	SIG	2300 ± 700
30	Barbituric acid ("6-hydroxyuracil")	SIG	†
31	6-Benzyluracil	MHK	†
32	6-Carboxyuracil (orotic acid)	SIG	†
33	6-Carboxymethyluracil (uracil-4-acetic acid)	SIG	†
34	6-Chlorouracil	SIG	†
35	6-Chloromethyluracil	ALD	†
36	6-Methylcarboxyuracil (methylorotate)	SIG	†
37	6-Methyluracil	SIG	†
38	6-Methylsulfonyluracil	SIG	†
	2,4-Substitutions		
39	4-Amino-2-thiouracil (2-thiocytosine)	SIG	‡
40	2-Chloropyrimidine ("2-chloro-4-deoxyuracil")	SIG	†
41	2,4-Dimethoxypyrimidine	SIG	†
42	2,4-Dithiouracil (2,4-dithiopyrimidine)	SIG	250 ± 60
43	4-Phenoxy-2-thiopyrimidine	SIG	‡
44	Pyrimidine ("2,4-dideoxyuracil")	SIG	†
45	2-Thiopyrimidine ("2-thio-4-deoxyuracil")	SIG	180 ± 30
	2,5-Substitutions		
46	5-Carbethoxy-2-thiouracil	SIG	1200 ± 400
47	5-Carboxy-2-thiouracil	SIG	1100 ± 300
48	5-Ethyl-2-thiouracil	SIG	‡
49	5-Methyl-2-thiouracil	SIG	‡
50	5-(*n*-Propyl)-2-thiouracil	SIG	2100 ± 600

Table 1 (continued)

	Compound	Source	AppK_i (μM)
	2,6-Substitutions		
51	6-Amino-2-thiouracil	SIG	‡
52	6-Benzyl-2-thiouracil	SIG	†
53	2-Methylmercaptobarbituric acid	ALD	‡
54	6-Methyl-2-thiouracil	SIG	1900 ± 700
55	6-(*n*-Propyl)-2-thiouracil	SIG	1700 ± 200
56	2-Thiobarbituric acid	SIG	†
	5,6-Substitutions		
57	5-Aminobarbituric acid (uramil)	SIG	†
58	5-Benzyloxybenzylbarbituric acid	MHK	†
59	5,6-Dihydrouracil	ALD	†
60	5-Isonitrosobarbituric acid (violuric acid)	ALD	†
61	5-Nitrobarbituric acid	SIG	†
62	5-Nitro-6-carboxyuracil (5-nitroorotic acid)	SIG	†
63	5-Nitro-6-methyluracil	SIG	‡
	Other substitutions		
64	6-Amino-5-nitroso-2-thiouracil	SIG	†
65	5-Bromo-1-methyluracil	SIG	†
66	4,6-Dihydroxypyrimidine ("5-aza-3-deazauracil")	SIG	†
Triazine compounds			
67	5-Azabarbituric acid (cyanuric acid)	ALD	‡
68	5-Azauracil	SIG	930 ± 320
69	6-Azauracil	SIG	†
70	6-Aza-2-thiouracil	AMB	†
71	1,3,5-Triazine ("5-aza-2,4-dideoxyuracil")	ALD	‡
72	Trithiocyanuric acid (2,4,6-trithio-5-azauracil)	ALD	110 ± 50
Pyrazine compounds			
73	2-Acetylpyrazine	SCH	‡
74	3-Amino-2-carboxypyrazine	SIG	†
75	2-Aminopyrazine	SCH	†
76	2-Carboxamidopyrazine	ALD	†
77	2-Carboxypyrazine	ALD	†
78	2-Chloropyrazine	SCH	‡
79	2-Ethylpyrazine	ALD	†
80	3-Hydroxypyrazine-1-oxide (emimycin)	ERP	270 ± 50
81	2-Methylpyrazine	SCH	†
82	2-Methoxypyrazine	SCH	‡
83	Pyrazine	SIG	†
Pyridine compounds			
84	3-Carboxypyridine-*N*-oxide	SCH	†
85	Citrazinic acid ("6-carboxy-1-deazauracil")	SIG	†
86	2,3-Dihydroxypyridine	SCH	†
87	2,4-Dihydroxypyridine ("3-deazauracil")	SIG	†
88	2,6-Dihydroxypyridine ("1-deazauracil")	ALD	200 ± 90
89	2-Hydroxypyridine	SCH	†
90	4-Hydroxypyridine	ALD	‡
91	2-Hydroxypyridine-*N*-oxide	ALD	†
92	3-Hydroxypyridine-*N*-oxide	ALD	†
93	Pyridine-*N*-oxide	ALD	†
94	2-Thiopyridine	SIG	570 ± 40
95	4-Thiopyridine	ALD	90 ± 33
96	2-Thiopyridine-*N*-oxide	SIG	1600 ± 700
Miscellaneous compounds			
97	1,3-Benzenediol (resorcinol; "1,3-dideazauracil")	SIG	†
98	Glutaric anhydride	ALD	‡
99	Glutarimide ("5,6-dihydro-1-deazauracil")	ALD	‡
100	3-Oxauracil	SIG	†

* AppK_i values ± the SD were obtained from at least three separate estimations of the appK_i.

† = less than 5% inhibition at a concentration of 2 mM.

‡ = less than 10% inhibition at a concentration of 2 mM.

Table 2. Ionization of pyrimidine, triazine, pyrazine and pyridine compounds

	Compound	pK_a	% Ionized at pH 8.5*	Ref.
Pyrimidine compounds				
1	Uracil	9.5	9	15
4	1-Methyluracil	9.7	6	15
5	1-Ribouracil (uridine)	9.3	14	14
6	2-Aminouracil (isocytosine)	9.6	7	15
7	4-Hydroxypyrimidine ("2-deoxyuracil")	8.6	44	16
8	2-Thiouracil	7.7	86	15
10	3-Methyluracil	9.9	4	15
11	4-Aminouracil (cytosine)	12.2	0	15
12	2-Hydroxypyrimidine ("4-deoxyuracil")	9.2	17	16
13	5-Aminouracil	9.5	9	17
15	5-Bromouracil	7.9	80	15
17	5-Carboxyuracil (isoorotic acid)	8.9	28†	15
18	5-Chlorouracil	8.0	76	15
21	5-Fluorouracil	8.0	76	15
22	5-Hydroxyuracil (isobarbituric acid)	8.1	72	18
23	5-Iodouracil	8.3	61	15
24	5-Methyluracil (thymine)	9.9	4	15
25	5-Nitrouracil	5.6	100	15
28	5-Trifluoromethyluracil	7.4	93	15
29	6-Aminouracil	8.7	39	17
30	Barbituric acid ("6-hydroxyuracil")	3.9	100	18
32	6-Carboxyuracil (orotic acid)	9.5	9†	15
34	6-Chlorouracil	5.7	100	15
36	6-Methylcarboxyuracil (methylorotate)	7.9	80	15
37	6-Methyluracil	9.7	6	15
38	6-Methylsulfonyluracil	4.7	100	15
39	4-Amino-2-thiouracil (2-thiocytosine)	10.6	1	15
42	2,4-Dithiouracil (2,4-dithiopyrimidine)	6.4	99	15
45	2-Thiopyrimidine ("2-thio-4-deoxyuracil")	7.1	96	15
51	6-Amino-2-thiouracil	7.3	94	15
55	6-(*n*-Propyl)-2-thiouracil	8.3	61	15
56	2-Thiobarbituric acid	7.9	80‡	15
60	5-Isonitrosobarbituric acid (violuric acid)	9.7	6‡	15
61	5-Nitrobarbituric acid	10.3	2	15
63	5-Nitro-6-methyluracil	6.4	99	17
65	5-Bromo-1-methyluracil	8.4	56	17
66	4,6-Dihydroxypyrimidine ("5-aza-3-deazauracil")	5.4	100	18
Triazine compounds				
67	5-Azabarbituric acid (cyanuric acid)	10.6	1‡	19
68	5-Azauracil	6.5	99	18
69	6-Azauracil	7.0	97	20
72	Trithiocyanuric acid (2,4,6-trithio-5-azauracil)	8.0	76‡	19
Pyrazine compounds				
80	3-Hydroxypyrazine-1-oxide (emimycin)	6.2	100	21
Pyridine compounds				
87	2,4-Dihydroxypyridine ("3-deazauracil")	6.5	99	18
88	2,6-Dihydroxypyridine ("1-deazauracil")	4.5	100	22
89	2-Hydroxypyridine	11.6	0	18
90	4-Hydroxypyridine	11.1	0	18
94	2-Thiopyridine	10.0	3	23
95	4-Thiopyridine	8.9	28	23

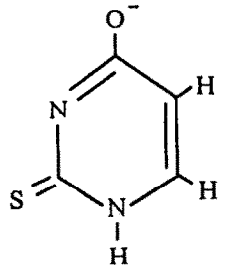
* Calculated using the equation: % Ionization = $(10^{(pH - pKa)}/1 + 10^{(pH - pKa)}) \times 100$.

† The carboxyl group is completely ionized at pH 8.5. The percent ionization figure refers to the dianion formed by dissociation of either the *N1* or *N3* proton.

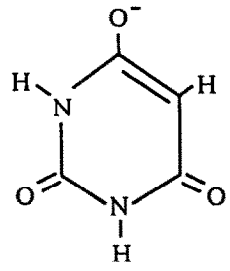
‡ All of these compounds are dianions. The percent ionization figure refers to the amount of dianion formed.

compounds 2-thiopyrimidine (**45**), 2,4-dithiouracil (**42**), and 2-thiouracil (**8**) all had measurable appK_i values, although the binding of these compounds was reduced compared with uracil. In addition, three pyridine compounds that have thio substitutions at either the 2- or 4-position (i.e. 2-thiopyridine, **94**; 4-thiopyridine, **95**; and 2-thiopyridine-*N*-oxide, **96**) were also able to bind to the enzyme.

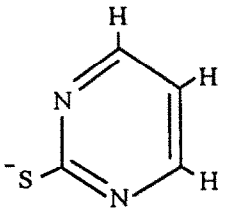
In general, substitution of a 2- or 4-position oxo group with a thio group increased the level of ionization of a compound (Table 2), regardless of the ring structure (e.g. pyrimidine and triazine).



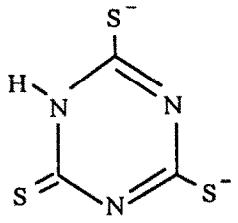
2-Thiouracil (**8**)



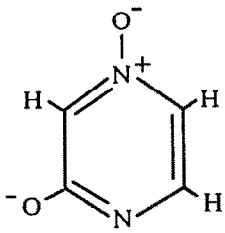
Barbituric acid (**30**)



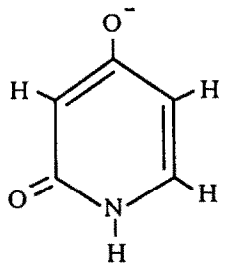
2-Thiopyrimidine (**45**)



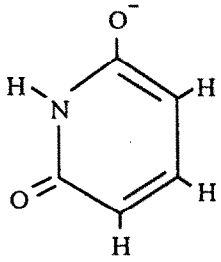
Trithiocyanuric acid (**72**)



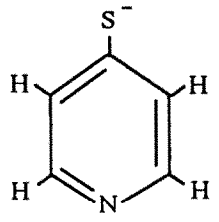
Emimycin (**80**)



3-Deazauracil (**87**)



1-Deazauracil (**88**)



4-Thiopyridine (**95**)

Fig. 2. Ionized forms of selected compounds (compound numbers from Table 1 shown in parentheses). References for these structures are as follows: 2-thiouracil and 2-thiopyrimidine [24]; barbituric acid and 4-thiopyridine [25]; 3-deazauracil [18]; 1-deazauracil [22]. The structure shown for trithiocyanuric acid is based on the ionization of cyanuric acid [26]. The structure shown for emimycin is one possible ionized form proposed by the authors.

However, the effect of this increase in ionization on binding to UPRTase varied depending on the type of compound. In the case of compounds with exocyclic groups at both the 2- and 4-positions, substitution of an oxo group with a thio group (e.g. 2-thiouracil, **8**; or 2,4-dithiouracil, **42**) decreased binding. For example, 2,4-dithiouracil bound to UPRTase 5-fold more poorly than uracil. This compound ionizes in a manner analogous to uracil (Fig. 1, panel B) in which there is a partial negative charge at both the 2- and 4-position exocyclic thio groups [24]; however, the level of ionization (Table 2) of 2,4-dithiouracil is much greater (99%) than that of uracil (9%). Thus, the decreased binding of

Table 3. Structure–activity relationship for the binding of ligands to *T. gondii* UPRTase

Position*	Substituent effect
N1	Endocyclic imine group not required for binding; replacement with methylene group (1-deazauracil, **88**) diminishes binding by only 4-fold.
	Exocyclic substituents larger than hydrogen (e.g. methyl, **4**) abolish binding, possibly due to overlap of substituents with the PRibPP binding site.
C2, C4	An exocyclic thio group at either the 2- or 4-position (e.g. 4-thiopyridine, **95**), or a combination of thio or oxo groups at both positions (e.g. 2,4-dithiouracil, **42**), required for binding.
	Negative charge on the exocyclic 4-position group may enhance binding; negative charge on the exocyclic 2-position group may diminish binding (e.g. ionized form of uracil shown in Fig. 1).
N3	Endocyclic imine group required for binding of compounds with exocyclic (oxo or thio) groups at both the 2- and 4-positions; replacement with methylene group (3-deazauracil, **87**) abolishes binding. Exception: emimycin (**80**) lacks an endocyclic imine group and binds only 5-fold less than uracil.
	Endocyclic imine group not required for binding of compounds with single exocyclic (oxo or thio) group at either the 2- or 4-position (e.g. 4-thiopyridine, **95**).
	Exocyclic substituents larger than hydrogen (e.g. methyl, **10**) abolish binding, possibly due to lack of *N3* hydrogen and/or steric hindrance.
C5	Endocyclic methylene group strongly preferred; replacement with an imine group (5-azauracil, **68**) decreases binding by 18-fold.
	Electron-withdrawing exocyclic substituents decrease (e.g. fluoro, **21**) or abolish (e.g. bromo, **15**) binding, possibly due to negative charge at the exocyclic 2-position.
	Exocyclic substituents larger than hydrogen (e.g. methyl, **24**) abolish binding, probably due to steric hindrance. Exception: diazo group (**19**) diminishes binding 25-fold.
C6	Endocyclic methylene group required; replacement with endocyclic imine group (6-azauracil, **69**) abolishes binding.
	Electron-withdrawing exocyclic substituents (e.g. chloro, **34**) abolish binding, possibly due to negative charge at the exocyclic 2-position.
	Exocyclic substituents larger than hydrogen (e.g. methyl, **37**) abolish binding, probably due to steric hindrance. Exception: amino group (**29**) diminishes binding 43-fold.
	Negatively charged exocyclic thio group may enhance binding (e.g. trithiocyanuric acid, **72**).

* Refers to pyrimidine ring numbering system shown in Fig. 1, panel A.

2,4-dithiouracil is consistent with the idea that the presence of negative charges at the 2- and 4-positions diminishes binding to UPRTase.

2-Thiouracil (**8**) bound to UPRTase 83-fold less than uracil. As with 2,4-dithiouracil (**42**), this compound is also highly ionized (86%) under the conditions employed (Table 2); however, ionization of 2-thiouracil occurs by dissociation of the *N3* proton, resulting in a negative charge localized on the exocyclic 4-position oxygen (Fig. 2). The poor binding of this compound is probably not due to the presence of a negative charge at the 4-position since 1-deazauracil (**88**) has a similar structure (Fig. 2) and binds to UPRTase with a greater affinity than 2-thiouracil. Similarly, the presence of a thio group at the 2-position probably does not decrease binding significantly given the appK_i for 2,4-dithiouracil. One possibility is that the *N3* proton plays an important role in the binding of compounds with thio or oxo groups at both the 2- and 4-positions. This may explain why 2,4-dithiouracil, which has an *N3* proton in one of its ionized forms (as shown in Fig. 1, panel B), binds better to UPRTase than does 2-thiouracil, which lacks the *N3* proton in its ionized form.

In the case of compounds with a single exocyclic group at either the 2- or 4-position (e.g. 2-hydroxypyrimidine, **12**; 2-hydroxypyridine, **89**; or 4-hydroxypyridine, **90**), substitution of an oxo group with a thio group (i.e. 2-thiopyrimidine, **45**; 2-thiopyridine, **94**; or 4-thiopyridine, **95**) increased binding to UPRTase. Ionization of these compounds occurs through the loss of either an *N1* or *N3* proton, resulting in a localized negative charge on the exocyclic thio group (Fig. 2). Thus, for these compounds it appears that the *N3* proton is not involved in binding to UPRTase, and a negative charge on either the 2- or 4-position exocyclic group enhances binding. However, this contradicts the idea that the presence of negative charges at the 2- and 4-positions diminishes binding to UPRTase (see above). One possible explanation is that the 2-thio-substituted compounds rotate, such that they bind to UPRTase with the exocyclic thio group in the 4-position. For example, 2-thiopyrimidine could bind in an orientation rotated 180° around the *N3-C6* axis as "4-thio-5-aza-1-deazapyrimidine." Therefore, it is possible that a negative charge at the exocyclic 4-position enhances binding to UPRTase, whereas a negative charge at the exocyclic 2-position diminishes binding. It should also be noted that the ionized forms of 2-thiopyrimidine and 4-thiopyridine are highly aromatic, with the π electrons distributed evenly throughout the ring (Fig. 2). Thus,

substitutions that increase the aromaticity of a compound may also enhance binding to UPRTase.

3-Position substitutions. In contrast to the 1-position endocyclic imine group, replacement of the 3-position endocyclic imine group with a methylene group ("3-deazauracil", **87**) abolished binding to UPRTase. However, 3-deazauracil is similar to 1-deazauracil (**88**) in that it is 99% ionized under the conditions employed (Table 2), and the ionized form of this compound has a negative charge localized at the exocyclic 4-position (Fig. 2). The only difference between these compounds is the position of the endocyclic imine group. Therefore, the lack of binding of 3-deazauracil supports the idea that the 3-position imine group plays a significant role in the binding of compounds with exocyclic (oxo or thio) groups at both the 2- and 4-positions. This is supported further by the fact that substitution of the *N3* hydrogen of uracil with either a butyl (**9**) or a methyl (**10**) group abolished binding, although the lack of binding may be due to steric hindrance at the 3-position. Replacement of the 3-position imine group with an endocyclic oxygen (3-oxauracil, **100**) also abolished binding to UPRTase; however, the lack of binding of 3-oxauracil may be due to the fact that the oxazine ring structure of this compound is "puckered" rather than planer [27].

5-Position substitutions. An endocyclic methylene group at the 5-position is strongly preferred for binding to UPRTase, as replacement with an imine group (5-azauracil, **68**) decreased binding by about 18-fold relative to uracil. 5-Azauracil is highly ionized (99%) under the conditions employed (Table 2), and ionization occurs through loss of the *N1* proton [25]. Thus, the ionized form of this compound is similar to that shown in Fig. 2 for 1-deazauracil (**88**) in which there is a localized negative charge on the exocyclic 4-position oxygen and *N3* is protonated. However, 1-deazauracil binds to UPRTase approximately 5-fold better than 5-azauracil. Since the ionization of these compounds is similar, it may be the presence of an endocyclic nitrogen at the 5-position of 5-azauracil that is responsible for the decreased binding of this compound relative to 1-deazauracil.

Exocyclic substituents at the 5-position that are electron-withdrawing decrease or abolish binding to UPRTase. For example, the fluorine atom of 5-fluorouracil (**21**) is similar to the 5-position hydrogen of uracil (**1**) with respect to its hydrophobic character and its van der Waals radius [12]. Thus, the decreased binding of 5-fluorouracil can be attributed to the electron-withdrawing properties of the fluorine atom, which increases the level of ionization of this compound relative to uracil (Table 2). Ionization of 5-fluorouracil occurs in a manner similar to that of uracil (Fig. 1, panel B); however, ionization occurs predominantly through loss of the *N3* proton rather than the *N1* proton [14]. Thus, the decreased binding of this compound supports the idea that the presence of negative charges at the 2- and 4-positions and the absence of an *N3* proton (for compounds with exocyclic groups at both the 2- and 4-positions) diminish binding to UPRTase.

In general, exocyclic substituents at the 5-position that are larger than a hydrogen atom abolish binding to UPRTase. For example, neither 5-aminouracil (**13**) nor 5-methyluracil (**24**) binds to UPRTase. These compounds are predominantly unionized under the conditions employed (Table 2), and neither of the substituents (amino, methyl) is charged. Furthermore, one substituent (amino) is hydrophilic while the other (methyl) is hydrophobic. Therefore, the effect of these substituents on binding to UPRTase cannot be explained based on their electron-withdrawing, hydrophilic or hydrophobic properties. The one parameter that these substituents have in common is that they are both larger than either a hydrogen or fluorine atom. Thus, it appears that UPRTase is very sterically hindered with respect to binding of compounds with 5-position substituents that are larger than the hydrogen found on uracil. One exception to this rule is the compound 5-diazouracil (**19**). Although the presence of a diazo group at the 5-position decreased binding approximately 25-fold relative to uracil, it is difficult to reconcile any binding to UPRTase considering the size of this group. It should be noted, however, that this group differs from all of the other substituents tested in that it contains a positive and a negative charge [28], which may have some unknown effect on binding.

6-Position substitutions. Replacement of the 6-position endocyclic methylene group with an imine group (6-azauracil, **69**) abolished binding to UPRTase. As with 5-azauracil (**68**), 6-azauracil is highly ionized under the conditions employed (Table 2); however, ionization of 6-azauracil occurs through loss of the *N3* proton rather than the *N1* proton, as is the case for 5-azauracil [25]. Therefore, the ionized form of 6-azauracil is similar to that shown in Fig. 2 for 2-thiouracil (**8**) in which the *N3* proton is missing and there is a negative charge on the exocyclic 4-position oxygen. Thus, the lack of binding of 6-azauracil may be due to the absence of an *N3* proton and the presence of an endocyclic nitrogen at the 6-position.

Exocyclic substituents at the 6-position that are either electron-withdrawing and/or larger than a hydrogen atom generally abolish binding to UPRTase. For example, barbituric acid (**30**) and 6-chlorouracil (**34**) are both highly ionized (Table 2) and neither binds to UPRTase. Ionization of 6-chlorouracil is similar to that of uracil (Fig. 1, panel B), whereas barbituric acid ionizes through loss of one of the *C5* hydrogens, resulting in a negative charge on the exocyclic 4-position oxygen (Fig. 2). Since the ionized form of barbituric acid is similar to that of 1-deazauracil (Fig. 2), which does bind to UPRTase, the lack of binding of barbituric acid may be the result of steric hindrance due to the presence of an exocyclic oxo group at the 6-position. This is supported by the fact that 6-methyluracil (**37**), which is predominantly unionized (Table 2), also does not bind to UPRTase. Thus, UPRTase may also be very sterically hindered with respect to binding of compounds with 6-position substituents that are larger than the hydrogen found on uracil. One exception to this generalization is the compound 6-aminouracil (**29**); however, the significance of 6-aminouracil binding to UPRTase is questionable, given the fact that it binds 43-fold less than uracil.

2,5-, 2,6- and 5,6-Substitutions. All of the 2,5-, 2,6- and 5,6-substituted compounds were either poor inhibitors (appK_i > 1 mM) of UPRTase or inhibited the enzyme less than 10% at a concentration of 2 mM. This is not surprising since none of the individual substitutions enhanced binding. In some cases, substitution of the 2-position oxo group of 5- or 6-substituted compounds (e.g. 5-propyluracil, **26**, or 6-methyluracil, **37**) with a thio group (i.e. 5-propyl-2-thiouracil, **50**; and 6-methyl-2-thiouracil, **54**) increased binding. This increase in binding may be due to the fact that these compounds ionize [24] in a manner similar to that of 2-thiouracil (**8**) in which there is a negative charge on the exocyclic 4-position oxygen (Fig. 2). However, the increase in binding of these compounds was marginal and did not occur in all cases (e.g. neither 5-methyluracil, **24**, nor 5-methyl-2-thiouracil, **49**, binds to UPRTase).

Other substitutions. Trithiocyanuric acid (**72**) was one of the best ligands (appK_i = 110 μM) of UPRTase identified in the present study. This compound is highly ionized under the conditions employed (Table 2) and is predominantly in the dianionic form in which there is a negative charge localized on two of the exocyclic thio groups (Fig. 2). The relatively good binding of this compound is difficult to reconcile given the poor binding of other compounds with similar features. Trithiocyanuric acid has an endocyclic imine group at the 3-position and a negatively charged exocyclic thio group at the 4-position, both of which appear to be important for binding (see above). However, this compound also has an endocyclic nitrogen at the 5-position, which should decrease binding as seen with 5-azauracil (**68**), and an exocyclic (thio) group at the 6-position, similar to the compound barbituric acid (**30**), which did not bind to UPRTase. One difference between trithiocyanuric acid and barbituric acid is that the 6-position (thio) group of trithiocyanuric acid is ionized, whereas the 6-position (oxo) group of barbituric acid is unionized (Fig. 2). Thus, it is possible that the negatively charged thio group at the 6-position of trithiocyanuric acid enhances binding. However, this may apply only to thio groups at the 6-position. For example, 2-thiobarbituric acid (**56**) has a negatively charged oxo group at the 6-position and does not bind to UPRTase.

Emimycin (3-hydroxypyrazine-1-oxide, **80**) binds to UPRTase about 5-fold less than uracil. Although this compound is a pyrazine, it can be considered to be an analog of uracil in which the endocyclic *N3* nitrogen is moved to the 4-position (i.e. "3-deaza-4-azauracil"). Emimycin is ionized 100% under the conditions employed (Table 2); however, the manner in which this compound ionizes is unknown. Since this compound has only one protonated nitrogen in the ring (*N1*), one possible ionization is through loss of this proton resulting in a negative charge on both the 2- and 4-position exocyclic oxygens (shown in Fig. 2). Thus, emimycin would resemble the ionized form of uracil (Fig. 1, panel B), except that the π electrons would be distributed evenly throughout the ring as is the case for 2-thiopyrimidine and 4-thiopyridine (Fig. 2). As noted above, an increase in the aromaticity of a compound may enhance binding to UPRTase. On the other hand, one feature of emimycin that is inconsistent with its ability to bind to UPRTase is the lack of an endocyclic imine group at the 3-position. All of the other compounds that lacked this group and had exocyclic oxo or thio groups at the 2- and 4-positions were either poor ligands or did not bind to UPRTase.

Evaluation of compounds as substrates for T. gondii *UPRTase*

Eight of the best ligands of *T. gondii* UPRTase identified in the present study were tested as substrates for this enzyme (i.e. uracil, **1**; 5-fluorouracil, **21**; 2,4-dithiouracil, **42**; 2-thiopyrimidine, **45**; trithiocyanuric acid, **72**; emimycin, **80**; 1-deazauracil, **88**; and 4-thiopyridine, **95**). Three of these compounds (uracil, 5-fluorouracil and emimycin) have been identified previously as substrates for this enzyme [6, 7] and were used as positive controls. When uracil, 5-fluorouracil and emimycin were tested, all three were converted to nucleotides in the presence of cytosol but not in its absence (results not shown), confirming that they are indeed substrates for UPRTase. Similar results (not shown) were seen for 2,4-dithiouracil, indicating that this compound is also a substrate for *T. gondii* UPRTase. In contrast, there was no difference between the HPLC profiles for 2-thiopyrimidine, trithiocyanuric acid, 1-deazauracil or 4-thiopyridine in the presence or absence of cytosol extract (results not shown). Thus, it would appear that these compounds are not substrates for *T. gondii* UPRTase under the conditions employed in the present study.

The four ligands of UPRTase identified as non-substrates for UPRTase (2-thiopyrimidine, trithiocyanuric acid, 1-deazauracil and 4-thiopyridine) have a single negative charge localized at either the 2- or 4-position exocyclic group (Fig. 2). In contrast, all four of the compounds identified as substrates of UPRTase (uracil, 5-fluorouracil, 2,4-dithiouracil and emimycin) have negative charges localized at both the 2- and 4-position exocyclic groups in their ionized form (Fig. 2). Thus, it appears that these features are required for the catalytic activity of *T. gondii* UPRTase, but not for binding to this enzyme.

Design of ligands of T. gondii *UPRTase*

Table 3 summarizes the structure–activity relationship for binding of ligands to *T. gondii* UPRTase, as determined from the results shown in Table 1. On the basis of these findings, several compounds (not currently available) can be proposed as potential ligands of UPRTase. For example, we would predict that 4-thiopyrimidine and 2-thio-1,3,5-triazine would be good ligands for UPRTase, based on the binding of 4-thiopyridine (**95**) and 2-thiopyrimidine (**45**). The pK_a for 2-thio-1,3,5-triazine is unknown; however, 4-thiopyrimidine is 98% ionized under the conditions employed [15]. Thus, these compounds would probably have a negatively charged thio group at the 4-position, which appears to enhance binding to UPRTase. In general, substitution of exocyclic oxo groups with thio groups increased the binding of compounds. Therefore, it would be of interest to test thio-derivatives of 1-deazauracil (2,6-dihydroxypyridine, **88**) and emimycin (3-hydroxy-

pyrazine-1-oxide, **80**), such as 2-hydroxy-6-thiopyridine, 2,6-dithiopyridine or 3-thiopyrazine-1-oxide, to see if they also bind to UPRTase. Similarly, 4,6-dithiopyrimidine and 2,4-dithio-1,3,5-triazine may also bind to UPRTase, since they would probably have negatively charged thio groups at the 4- and 6-positions, as does trithiocyanuric acid (**72**).

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