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Pyrimidine Nucleobase Ligands of Orotate Phosphoribosyltransferase from *Toxoplasma gondii*

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ABSTRACT. Sixty-seven pyrimidine nucleobase analogues were evaluated as ligands of *Toxoplasma gondii* orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) by measuring their ability to inhibit this enzyme *in vitro*. Apparent *Ki* values were determined for compounds that inhibited *T. gondii* OPRTase by greater than 20% at a concentration of 400 μM. 1-Deazaorotic acid (0.47 μM) and 5-azaorotic acid (2.1 μM) were found to bind better (8.3- and 1.9-fold, respectively) to *T. gondii* OPRTase than orotic acid, the natural substrate of the enzyme. Based on these results, a structure–activity relationship of ligand binding to OPRTase was formulated using uracil, barbituric acid, and orotic acid as reference compounds. It was concluded that the following structural features of pyrimidine nucleobase analogues were required or strongly preferred for binding: (i) an endocyclic pyridine-type nitrogen or methine at the 1-position; (ii) exocyclic oxo groups at the 2- and 4-positions; (iii) a protonated endocyclic pyridine-type nitrogen at the 3-position; (iv) an endocyclic pyridine-type nitrogen or methine at the 5-position; (v) an exocyclic hydrogen or fluorine at the 5-position; (vi) an endocyclic pyridine-type nitrogen or methine at the 6-position; and (vii) an exocyclic negatively charged or electron-withdrawing group at the 6-position. A comparison of the results from the present study with those from a previous study on mammalian OPRTase [Niedzwicki *et al.*, *Biochem Pharmacol* 33: 2383–2395, 1984] identified four compounds (6-chlorouracil, 5-azaorotic acid, 1-deazaorotic acid, and 6-iodouracil) that may bind selectively to *T. gondii* OPRTase. BIOCHEM PHARMACOL **58**;9:1457–1465, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *Toxoplasma gondii*; orotate phosphoribosyltransferase; pyrimidine nucleobases; structure-activity

Toxoplasma gondii is an obligate intracellular parasitic protozoan that causes the disease toxoplasmosis. Although *Toxoplasma* infections are mostly asymptomatic (90% of cases) in immunocompetent individuals [1], immunocompromised hosts (e.g. individuals with AIDS) are at great risk from *T. gondii* infections. It has been shown that of all the opportunistic infections in AIDS patients, one third are *Toxoplasma* infections [2, 3]. Furthermore, *T. gondii* is the most common opportunistic infection of the central nervous system (toxoplasmic encephalitis) in these patients [4], and it has been suggested that approximately 26% of HIV¶-positive individuals in the U.S.A., previously exposed to *Toxoplasma*, will contract toxoplasmic encephalitis [5]. Congenital toxoplasmosis occurs in approximately 1/1000 live births [6], and its effects range in severity from asymptomatic infection to stillbirth, with the most common ailments being retinochoroiditis and cerebral calcifications [7].

The current standard treatment for toxoplasmosis involves a combination of pyrimethamine and sulfadiazine [8], which results in clinical improvement in approximately 80–90% of patients within 2–21 days [9]. Nevertheless, toxic side-effects occur in greater than 40% of these patients [10, 11]. Alternative treatments have been investigated, such as clindamycin with pyrimethamine; however, the toxicity and efficacy of these therapies are similar to those shown for the standard treatment [12]. Thus, it is imperative that alternative drugs be available for patients who develop toxicity from the standard treatments [2].

T. gondii divide rapidly and, therefore, need large amounts of pyrimidine nucleotides for the synthesis of nucleic acids. Consequently, pyrimidine nucleotide metabolism is a potential site for chemotherapeutic intervention. Indeed, two pyrimidine analogues, 5-fluorouracil [13] and emimycin (3-deaza-4-azauracil) [14], have been shown to inhibit *T. gondii* replication by more than 90%. Pyrimidine metabolism in *T. gondii* occurs by the salvage [13] and/or *de novo* pathways [15]. Previous studies have focused on inhibition of the pyrimidine salvage enzymes uridine phosphorylase (EC 2.4.2.3) [16, 17] and UPRTase (EC 2.4.2.9) [18]. However, to block pyrimidine biosynthesis in *Toxo-*

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¶ *Abbreviations*: HIV, human immunodeficiency virus; OPRTase, orotate phosphoribosyltransferase; PRibPP, 5-phosphoribosyl-1-pyrophosphate; and UPRTase, uracil phosphoribosyltransferase.

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TABLE 1. Apparent K_i values for inhibition of *T. gondii* OPRTase

Compound		Source	Apparent K_i* (μM)
Uracil analogues			
1	Uracil	SIG	340 ± 20
	1-Substitutions		
2	1-Deazauracil (2,6-dihydroxypyridine)	ALD	350 ± 310
3	1-Methyluracil	SIG	†
4	1-Cyclohexyluracil	SIG	†
5	1-Benzyluracil	VEGA	†
	2-Substitutions		
6	4-Hydroxypyrimidine (2-deoxyuracil)	SIG	†
7	2-Aminouracil (isocytosine)	SIG	†
8	2-Thiouracil	SIG	1030 ± 400
	3-Substitutions		
9	3-Deazauracil (2,4-dihydroxypyridine)	SIG	1730 ± 30
10	3-Oxauracil	SIG	†
11	3-Methyluracil	SIG	†
12	3-Butyluracil	SIG	†
	4-Substitutions		
13	2-Hydroxypyrimidine (4-deoxyuracil)	SIG	†
14	4-Aminouracil (cytosine)	SIG	†
	5-Substitutions		
15	5-Azauracil	SIG	270 ± 100
16	5-Fluorouracil	SIG	200 ± 130
17	5-Chlorouracil	SIG	850 ± 20
18	5-Bromouracil	SIG	†
19	5-Iodouracil	SIG	†
20	5-Trifluoromethyluracil	SIG	†
21	5-Aminouracil	SIG	†
22	5-Carbethoxyuracil	SIG	†
23	5-Carboxyuracil (isoorotic acid)	SIG	†
24	5-Diazouracil	SIG	470 ± 130
25	5-Hydroxyuracil (isobarbituric acid)	SIG	†
26	5-Nitrouracil	SIG	460 ± 100
27	5-Sulfaminouracil	SIG	†
28	5-Methyluracil (thymine)	SIG	†
29	5-Ethyluracil	SIG	†
30	5-(*n*-Propyl)uracil	SIG	†
31	5-Benzyluracil	SHC	†
	6-Substitutions		
32	6-Azauracil	SIG	330 ± 80
33	6-Chlorouracil	SIG	26 ± 10
34	6-Iodouracil	VEGA	53 ± 13
35	6-Chloromethyluracil	ALD	320 ± 60
36	6-Aminouracil	SIG	110 ± 50
37	6-Carboxymethyluracil (uracil-4-acetic acid)	SIG	260 ± 70
39	6-Methyluracil	SIG	†
40	6-Benzyluracil	SHC	†

Continued

TABLE 1. Continued

Compound		Source	Apparent K_i* (μM)
	Multiple substitutions		
41	2,4-Dithiouracil (2,4-dithiopyrimidine)	SIG	†
42	Trithiocyanuric acid (2,4,6-trithio-5-azauracil)	ALD	65 ± 16
43	3-Hydroxypyrazine-1-oxide (3-deaza-4-azauracil; emimycin)	ERP	†
44	4,6-Dihydroxypyrimidine (5-aza-3-deazauracil)	SIG	340 ± 20
45	5-Nitro-6-methyluracil	SIG	†
Barbituric acid analogues			
46	Barbituric acid (6-hydroxyuracil)	SIG	20 ± 1.0
47	1,3-Dimethylbarbituric acid	ALD	†
48	2-Thiobarbituric acid	SIG	41 ± 10
49	5-Aminobarbituric acid (uramil)	SIG	†
50	5-Azabarbituric acid (cyanuric acid)	ALD	220 ± 100
51	5-Ethyl-5-(*p*-hydroxyphenyl) barbituric acid	SIG	†
52	5-Isonitrosobarbituric acid (violuric acid)	ALD	†
53	5-Nitrobarbituric acid	SIG	†
Orotic acid analogues			
54	Orotic acid (6-carboxyuracil)	SIG	3.9 ± 0.9
55	1-Deazaorotic acid (4-carboxy-2,6-dihydroxypyridine; citrazinic acid)	SIG	0.47 ± 0.30
56	Orotidine (1-riboorotic acid)	SIG	280 ± 10
57	2-Thioorotic acid	VEGA	220 ± 10
58	2-Methylthioorotic acid	ALD	140 ± 20
59	5-Azaorotic acid (oxonic acid)	SIG	2.1 ± 0.7
60	5-Aminoorotic acid	SIG	†
61	5-Fluoroorotic acid	SIG	16 ± 2.0
62	5-Bromoorotic acid	SIG	57 ± 14
63	5-Iodoorotic acid	SIG	140 ± 20
64	5-Nitroorotic acid	SIG	1100 ± 100
65	6-Methylorotate (orotic acid methylester)	SIG	7.0 ± 2.0
66	DL-Dihydroortotic acid	SIG	1200 ± 50
67	L-Dihydroortotic acid	SIG	810 ± 60

*Apparent K_i values ± SD were obtained from at least three separate estimations of the apparent K_i.
†Less than 20% inhibition at a concentration of 400 μM.

plasma completely, both the *de novo* and the salvage pathways need to be inhibited.

In a search for inhibitors of *de novo* pyrimidine biosynthesis in *T. gondii*, we focused our studies on the enzyme OPRTase (EC 2.4.2.10). OPRTase catalyzes the fifth step in the *de novo* pyrimidine pathway, and numerous potential ligands are available for testing. In the present investigation, we evaluated 67 pyrimidine nucleobase analogues as ligands of *T. gondii* OPRTase by determining their ability to inhibit its activity *in vitro*. Apparent K_i values were determined for all compounds that inhibited OPRTase by greater than 20% at a concentration of 400 μM. An analysis of these results led to the development of a structure–activity relationship, which identified the chemical properties of analogues that increased or decreased ligand binding to OPRTase. In addition, these results were compared with those reported for mammalian OPRTase [19] to identify analogues that would bind preferentially to the parasite enzyme.

MATERIALS AND METHODS

Chemicals and Supplies

Sources for compounds used as inhibitors of *T. gondii* OPRTase are listed in Table 1 as follows: ALD, the Aldrich Chemical Co.; ERP, Dr. Elmer R. Pfefferkorn, Department of Microbiology, Dartmouth Medical School; SHC, Dr. Shih-Hsi Chu, Division of Biology and Medicine, Brown

University; SIG, the Sigma Chemical Co.; and VEGA, Vega Biochemicals. [2-^{14}C]Orotic acid (55 Ci/mol) and [5-^{3}H]orotic acid (10 Ci/mmol) were obtained from Moravek Biochemicals, Inc.; scintillation fluid (ScintiLene) was purchased from Fisher Scientific; and cellulose 300 PEI/UV_{254} Polygram thin-layer chromatography plates were obtained from Brinkmann Instruments, Inc. All other chemicals and compounds used in this study were purchased from Fisher Scientific or Sigma.

Preparation of Enzyme Extracts

Tachyzoites of the RH strain of *T. gondii* were propagated by i.p. passage in female Swiss-Webster mice (Sasco, Inc.) [13], and cytosol extracts were prepared as previously described [18].

Determination of OPRTase Assay Conditions

Tris-Cl buffer at pH 8.5 was the optimal buffer for *T. gondii* OPRTase activity. The apparent K_m value for orotate was estimated to be 1.7 ± 0.2 μM using [5-^{3}H]orotic acid (10 Ci/mmol) as a substrate at 0.75 to 5.0 μM. Therefore, 2 μM orotic acid was used for the inhibition studies at a PRibPP concentration of 100 μM, which was found to be saturating under the conditions employed. In addition, enzyme velocity was linear up to 40 μL of cytosol extract and 20 min of incubation time at 37°.

OPRTase Assays

OPRTase activity was determined by measuring the formation of radiolabeled OMP, UMP, orotidine, uridine, and uracil from the substrates [2-^{14}C]orotic acid and PRibPP. The reaction mixture contained 2 μM [2-^{14}C]orotic acid (55 Ci/mol), 100 μM PRibPP, 5 mM $MgCl_2$, 50 mM Tris–Cl buffer (pH 8.5), 1 mM dithiothreitol, 15–40 μL of enzyme extract, and a 0, 50, 100, 200, or 400 μM concentration of the analogue to be tested in a final volume of 150 μL. Higher concentrations of the test compound were used when testing poor inhibitors, whereas lower concentrations were used for potent inhibitors. Reactions in triplicate were initiated by the addition of [2-^{14}C]orotic acid, incubated at 37° for 20 min, and terminated by boiling in a water bath for 3 min and freezing at −20°. Precipitated proteins were removed by centrifugation, and a 15-μL aliquot of the supernatant was mixed with 5 μL of a standard solution (10 mM orotic acid, UMP, uridine, and uracil, and 5 mM OMP and orotidine). These mixtures were spotted on PEI-cellulose TLC plates (predeveloped with water), which then were developed with 0.2 M LiCl for approximately 2.5 hr. The R_f values were: OMP, 0.03; UMP, 0.17; orotic acid, 0.47; orotidine, 0.66; uridine, 0.79; and uracil, 0.86. Substrate and product spots were identified by UV quenching and cut out, and the radioactivity was quantitated by liquid scintillation counting in 20 mL of scintillation fluid (ScintiLene) in a Packard 460 scintillation counter.

Calculations and Statistical Analysis

Enzyme velocity was calculated by multiplying the fraction of products (OMP, UMP, orotidine, uridine, and uracil) formed from orotate times the amount of orotate in the assay, and dividing by the incubation time. Apparent K_m and apparent K_i values were estimated from Lineweaver–Burk plots ($1/v$ versus $1/[S]$) and Dixon plots ($1/v$ versus $[I]$) of the data, respectively, using computer programs developed by Dr. Sungman Cha (Brown University) and Dr. Fardos N. M. Naguib (University of Alabama at Birmingham) that employ the general principles of Cleland [20]. If a compound is a competitive inhibitor with respect to orotate, apparent K_i values are related to *Ki* values by the following equation [19]: apparent $K_i = K_i\ (1 + [S]/K_m)$. In the present study, the concentration of orotate (2 μM) was approximately equal to its K_m value (1.7 μM), and the concentration of PRibPP (100 μM) was a saturating concentration. Thus, the apparent K_i value determined for a competitive inhibitor would be about 2-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 [21] using the Bio-Rad Laboratories protein assay kit and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Evaluation of Pyrimidine Analogues as Ligands of T. gondii *OPRTase*

The analogues screened in this study were divided into three categories: uracil, barbituric acid, and orotic acid analogues. Ideally, only orotic acid analogues would have been used in this study; however, there were insufficient orotic acid analogues available to complete a structure–activity study on OPRTase. Therefore, other analogues were used, including a large number of uracil analogues that were available. Furthermore, during the course of this study, it was found that barbituric acid bound fairly well to the enzyme. Therefore, barbituric acid analogues also were tested to ascertain their effect on the enzyme. These analogues were evaluated as ligands of *T. gondii* OPRTase by examining their ability to inhibit this enzyme *in vitro*. Apparent K_i values were determined for those compounds that inhibited OPRTase by greater than 20% at a concentration of 400 μM. The apparent K_i values of these compounds are shown in Table 1.

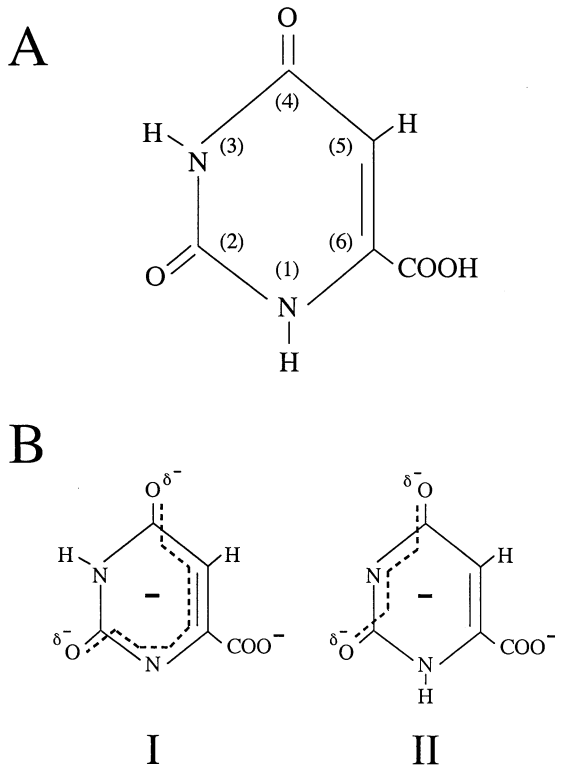

FIG. 1. Tautomerism and ionization of orotic acid. Panel A shows the diketo tautomeric form of orotic acid, which probably predominates in solution, and the pyrimidine numbering system. Panel B shows the probable dianionic structure of orotic acid formed by loss of either the *N1* (I) or *N3* (II) proton [23].

Tautomerism and Ionization of Orotic Acid

Orotic acid is the natural substrate for *T. gondii* OPRTase; therefore, the tautomerism and ionization of orotic acid may lend insight into how it binds to the enzyme. No specific information on the tautomerism of orotic acid (6-carboxyuracil) is available in the literature. However, all uracils, thymines, and their derivatives have been observed to exist in the diketo form [22, 23]. Hence, it is very likely that orotic acid also predominates in solution in this form (Fig. 1A). Ionization of orotic acid occurs by dissociation of a proton from the *N*1 or *N*3 endocyclic nitrogen and from the C6 exocyclic carboxyl group. If orotic acid ionizes in a manner similar to uracil, then the electrons from the dissociation of the *N*1 or *N*3 proton are delocalized, as shown in Fig. 1B. It should be noted that this process is pH dependent. Under the conditions of the present study (pH 8.5), the *N*1 or *N*3 proton of orotic acid dissociates only 9% (in an approximately 1:1 ratio) [23], while the carboxyl group is ionized completely (Fig. 2) [24]. Therefore, delocalization is of minor importance, and it is very likely that *T. gondii* OPRTase preferentially binds the monoanionic form of orotic acid rather than the dianionic form.

Binding of Compounds to T. gondii *OPRTase*

The results shown in Table 1 will be discussed based on substituent effects at the various positions of the pyrimidine ring (Fig. 1A) on binding to *T. gondii* OPRTase. To simplify the discussion, the pyrimidine ring numbering system will be used for all compounds (Fig. 1A). Inhibitors will be referred to by name and number (in bold type) from Table 1.

1-position substitutions. A pyridine-type nitrogen is not required for binding, as replacement with a methine group did not affect binding to *T. gondii* OPRTase markedly. Two "1-deaza" compounds (1-deazauracil, **2**; 1-deazaorotic acid, **55**) bound as well as or better than their respective unsubstituted compounds, uracil (**1**) and orotic acid (**54**). In fact, 1-deazaorotic acid (**55**) was the best ligand (0.47 μM) found in this study, as it bound 8.3-fold better to OPRTase than orotic acid, the natural substrate of the enzyme (**54**). Unfortunately, no pK_a value or ionization pattern is available for this analogue. However, it is likely that 1-deazaorotic acid (**55**) ionizes in a manner analogous to 1-deazauracil (**2**), which has been shown [18] to ionize at the C4 exocyclic oxygen rather than the *N*3 proton, resulting in a negative charge localized on the exocyclic 4-position oxygen (Fig. 2). In addition, 1-deazauracil (**2**) is 100% ionized [18] under the conditions of the present study. Thus, the increased ionization of "1-deaza compounds," resulting in a negative charge on the exocyclic 4-position oxygen, may improve binding of these compounds. In any event, the present results indicate that hydrogen bonding is not occurring or is not a significant factor in binding between the endocyclic *N*1 (either protonated or unprotonated) and the active site of *T. gondii* OPRTase.

The addition of hydrophobic exocyclic groups at the 1-position (methyl, **3**; cyclohexyl, **4**; benzyl, **5**) abolished binding to the enzyme. The lack of binding of these compounds is probably not due to the absence of the *N*1 proton, given the binding of the "1-deaza" compounds (see above), but may be due to steric hindrance by the large hydrophobic groups at the 1-position. Another possibility is that the reaction mechanism for *T. gondii* OPRTase is such that PRibPP binds to the enzyme before orotic acid. Therefore, the addition of exocyclic groups larger than hydrogen at *N*1 would overlap with the PRibPP binding site and abolish binding. One exception was 1-riboorotic acid (orotidine, **56**), which was able to bind to OPRTase, albeit 71-fold less than orotic acid. Binding of this compound may be due to its similarity to the reaction product, orotidine-5′-monophosphate.

2- and 4-position substitutions. An exocyclic oxo (hydroxy) group at both C2 and C4 is strongly preferred for binding, as elimination of an oxo group (e.g. 4-hydroxypyrimidine, **6**; 2-hydroxypyrimidine, **13**) or substitution with an amino group (2-aminouracil, **7**; 4-aminouracil, **14**) at either the C2 or C4 abolished binding. The poor binding of the aminouracils may be due to several reasons. First, an amino group is unable to form hydrogen bonds in the same manner as an oxo group. Second, an amino group could

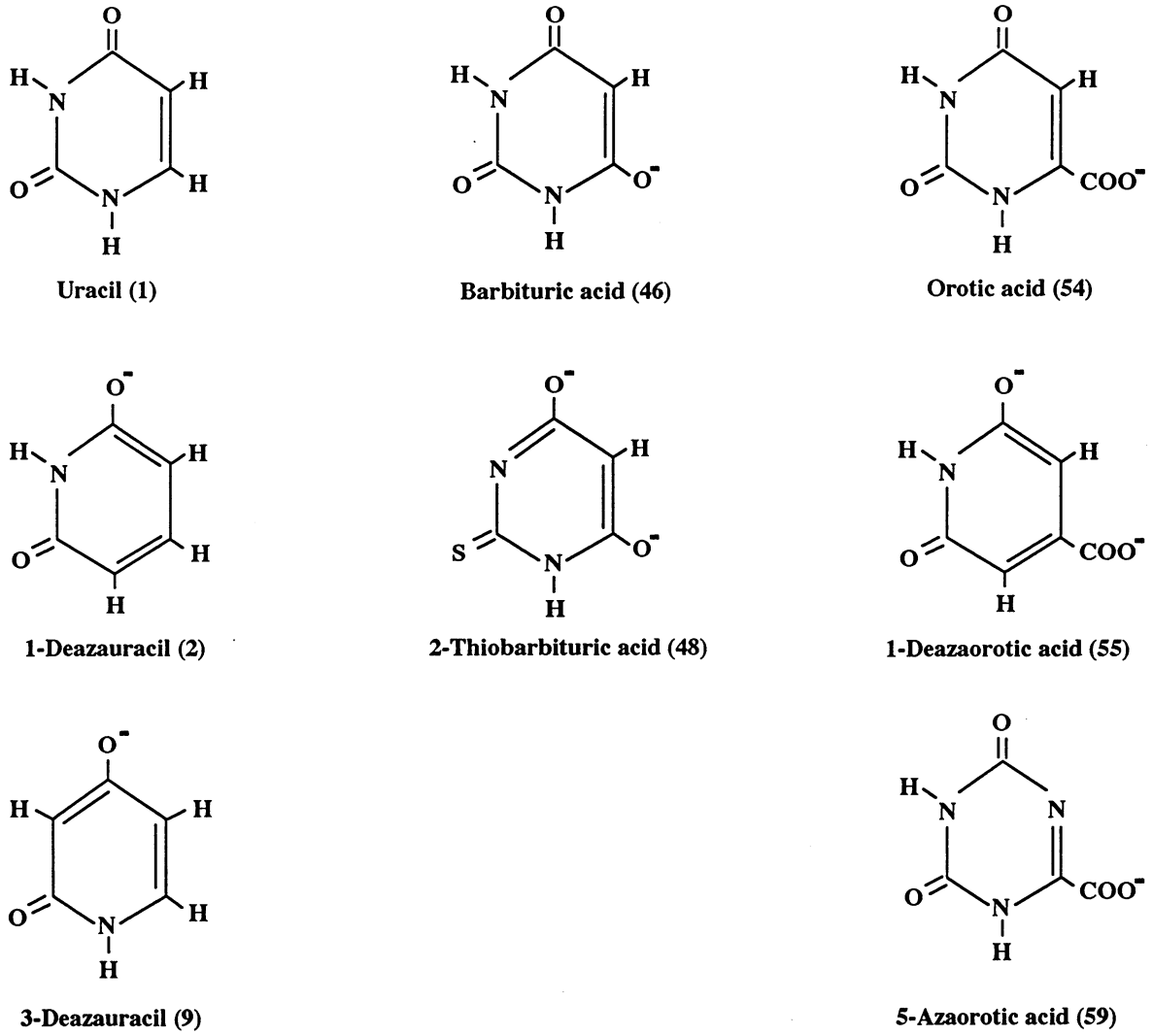

FIG. 2. Ionized forms of selected compounds. Numbers in parentheses are compound numbers from Table 1. References for these compounds are as follows: uracil [23]; 1-deazauracil [24]; 3-deazauracil [25]; barbituric acid [22]; and 2-thiobarbituric acid [26]. The structure shown for orotic acid is based on the tautomeric form of uracil [23], and the ionization of orotic acid [26]. The structure shown for 1-deazaorotic acid is based on ionization of both 1-deazauracil [24] and orotic acid [26]. The structure shown for 5-azaorotic acid is based on the ionization of both 5-azauracil [25] and orotic acid [26].

cause steric hindrance. Third, 4-aminouracil lacks a proton at *N*3 [23], which appears to play an important role in binding (see below). Although the manner of ionization of 2-aminouracil is unknown, it is certainly conceivable that it lacks binding for the same reason. These results suggest that the oxo groups help align the ligand with the catalytic site of the enzyme through hydrogen bonding.

An exocyclic thio group substitution at C2 (2-thiouracil, **8**; 2-thiobarbituric acid, **48**; 2-thioorotic acid, **57**) decreased the binding of uracil (**1**), barbituric acid (**46**) and orotic acid (**54**) (3.1-, 2.1-, and 56-fold, respectively), while substitution of thio groups at both C2 and C4 (2,4-dithiouracil, **41**) abolished binding. Substitution of exocyclic thio groups at the C2 and/or C4 position of pyrimidine compounds increases their ring ionization [18]. In general, increased ring ionization improves the binding of pyrimidine analogues to *T. gondii* OPRTase (see below, 6-Position Substitutions), presumably due to an increase in the negative charge on the exocyclic groups at C2 and C4. Thus, the decreased binding of the thio-substituted compound suggests that some other factor is involved. One possibility is that ionization of these compounds results in the dissociation of the *N*3 proton, which may play an important role in hydrogen bonding to the catalytic site (see 3-Position Substitutions). In addition, the larger size of the sulfur or its decreased electronegativity relative to oxygen may result in diminished binding to *T. gondii* OPRTase due to steric hindrance or decreased hydrogen bonding.

3-POSITION SUBSTITUTIONS. In contrast to the *N*1 pyridine-type nitrogen, the *N*3 pyridine-type nitrogen appears to play a significant role in the binding of ligands to *T. gondii* OPRTase. Replacement of the 3-position pyridine-

type nitrogen with a methine group (3-deazauracil, **9**) decreased binding 5-fold, whereas replacement with an endocyclic oxygen (3-oxauracil, **10**) abolished binding. The lack of binding of 3-oxauracil can be explained by the fact that the oxazine ring of 3-oxauracil is "puckered" rather than planar as is the case for the pyrimidine ring of uracil (**1**) [27]. The decreased binding of 3-deazauracil (**9**), on the other hand, probably is due to the lack of an *N*3 proton. Both 1-deazauracil (**2**) and 3-deazauracil (**9**) are highly ionized (100 and 99%, respectively) under the conditions of this study, and ionization of both compounds results in a negative charge at the 4-position exocyclic oxygen [18] (Fig. 2). The major difference between 1-deazauracil (**2**) and 3-deazauracil (**9**) is the position of the protonated imine group (*N*3 for 1-deazauracil, **2**; *N*1 for 3-deazauracil, **9**). Therefore, the differences in binding between these compounds suggest that the *N*3 proton (but not the *N*1 proton) is important for binding, probably through hydrogen bonding with a negatively charged amino acid at the catalytic site of OPRTase.

There are several additional examples that support the importance of the *N*3 proton for binding. First, the best ligands identified in this study (e.g. 6-chlorouracil, **33**; barbituric acid, **46**; orotic acid, **54**; 1-deazaorotic acid, **55**; and 5-azaorotic acid, **59**) all have an *N*3 proton (Fig. 2). Second, compounds that lack the *N*3 proton bound poorly or did not bind at all (e.g. 2-aminouracil, **7**; 2-thiouracil, **8**; 4-aminouracil, **14**; 3-deaza-4-azauracil, **43**; 2-thiobarbituric acid, **48**; 2,4-dithiouracil, **41;** and 2-thioorotic acid, **57**). Third, binding was abolished when the hydrogen at *N*3 was replaced with an exocyclic hydrophobic substituent (e.g. methyl, **11**; or butyl, **12**). One exception to this generalization was 5-azabarbituric acid (**50**). Although 5-azabarbituric acid (**50**) and 2,4,6-trithio-5-azauracil (**42**) are similar in structure and both have an *N*3 proton, the former (**50**) did not bind as well to the enzyme. The reason for this lack of binding by 5-azabarbituric acid (**50**) is unclear.

5-position substitutions. The endocyclic methine group at the 5-position is not required for binding to *T. gondii* OPRTase. Replacement of the C5 endocyclic methine group of uracil (**1**) and orotic acid (**54**) with an endocyclic nitrogen (e.g. 5-azauracil, **15**; or 5-azaorotic acid, **59**) increased binding slightly (1.3- and 1.9-fold, respectively), whereas a similar substitution to barbituric acid (**46**) (i.e. 5-azabarbituric acid, **50**) decreased binding by 11-fold. 5-Azauracil (**15**) is highly ionized (99%) under the conditions employed [18], and the ionized form of this compound [25] is similar to that shown in Fig. 2 for 1-deazauracil (**2**). The ionization processes for 5-azaorotic acid (**59**) and 5-azabarbituric acid (**50**), on the other hand, are unknown. Based on the relatively good binding of 5-azauracil (**15**) and 5-azaorotic acid (**59**), it is probable that the ionization of 5-azaorotic acid is similar to that for 5-azauracil, in which the *N*3 proton is retained. In contrast, the poor binding of 5-azabarbituric acid (**50**) suggests that this compound ionizes in a different manner, possibly through the loss of the *N*3 proton, as is the case for 2-thiobarbituric acid (**48**).

With the exception of the addition of a fluoro group to uracil (**1**) (i.e. 5-fluorouracil, **16**), all exocyclic substitutions at C5 decreased or abolished binding relative to the parent compound. This included halogens (e.g. chloro, **17**; bromo, **18**, **62**; and iodo, **19**, **63**), hydrophobic groups (e.g. trifluoromethyl, **20**; methyl, **28**; ethyl, **29**; *n*-propyl, **30**; benzyl, **31**; and ethyl-(*p*-hydroxyphenyl), **51**), and hydrophilic groups (e.g. amino, **21**, **49**, **60**; carbethoxy, **22**; carboxy, **23**; hydroxy, **25**; isonitroso, **52**; nitro, **45**, **53**, **64**; sulfamino, **27**). Compounds with halogen substituents at the 5-position displayed an inverse relationship between the size of the substituent and their ability to bind. For example, the binding of uracil analogs (**1**) with 5-substituted halogens decreased with an increase in the size of the halogen (i.e. 5-fluorouracil, **16** > 5-chlorouracil, **17** > 5-bromouracil, **18** = 5-iodouracil, **19**). This pattern was also seen when a halogen was added to orotic acid (5-fluoroorotic acid, **61** > 5-bromoorotic acid, **62** > 5-iodoorotic acid, **63**). Similarly, the poor binding of ligands with exocyclic hydrophobic substitutions at C5 is most likely due to steric hindrance. This is exemplified best by 5-methyluracil (**28**), which is similar to uracil (**1**) with respect to its ionization [18] and structure, and yet the addition of a methyl group to uracil abolished binding. Hydrophilic substitutions at C5 (e.g. amino, **21**, **49**, **60**; carbethoxy, **22**; carboxy, **23**; hydroxy, **25**; isonitroso, **52**; nitro, **45**, **53**, **64**; sulfamino, **27**) also significantly decreased or abolished binding. There were, however, two exceptions to this general trend. Replacement of the exocyclic C5 hydrogen with a diazo (5-diazouracil, **24**) or nitro group (5-nitrouracil, **26**) decreased binding by only 1.4-fold. This was surprising, since both the diazo and nitro groups are larger than hydrogen. Furthermore, 5-diazouracil (**24**) is in fact 5-diazo-1,6-dihydropyrimidin-2,4(1*H*,3*H*,6*H*)dione [28], where the *N*3 is unprotonated and the pyrimidine ring is unionized. These results suggest that some other factor(s) is involved. The diazo group, unlike any other substituent tested, has a positive and negative charge at the exocyclic 5-position [28], which may have some unknown effect on binding. A similar finding was observed in an analogous study of UPRTase [18].

Fluorine is similar to hydrogen in size [29] and hydrophobicity [30] but is more electronegative. The electron-withdrawing properties of fluorine are believed to cause an increase in pyrimidine ring ionization. For example, under our assay conditions, 5-fluorouracil (**16**) would be 76% ionized, while uracil (**1**) would be only 9% ionized [18]. However, this large increase in ionization was not accompanied by a large increase in binding (1.7-fold) of 5-fluorouracil (**16**) when compared with uracil (**1**). This could be due to the fact that 5-fluorouracil (**16**) ionizes in a manner such that approximately two-thirds of the ionized form is deprotonated at *N*3 [23], which has been shown to decrease binding (see above). Thus, the key to increased binding of ligands to *T. gondii* OPRTase appears to be increased ionization of the pyrimidine ring at the proper and specific locations. Similar arguments can be used to

explain the decreased binding (4.1-fold) of 5-fluoroorotic acid (**61**) when compared with orotic acid (**54**). The addition of a fluoro group at C5 may also affect the pK_a of the carboxyl group at C6 sufficiently to decrease the binding of 5-fluoroorotic acid (**61**).

6-position substitutions. An endocyclic carbon at the 6-position is not required for binding, as replacement with an endocyclic nitrogen (6-azauracil, **32**) did not affect binding. It is of interest to note that 6-azauracil is highly (97%) ionized under the conditions of the present study [18]; however, ionization occurs through loss of the *N*3 proton [22]. Thus, 6-azauracil is another example of a compound that is highly ionized but does not bind better than uracil (**1**) to OPRTase.

Exocyclic substitutions at the 6-position, which increase pyrimidine ring ionization (i.e. electron-withdrawing), also increase binding. For example, substitution of an amino (6-aminouracil, **36**), iodo (6-iodouracil, **34**), or chloro group (6-chlorouracil, **33**) at the 6-position of uracil increased binding by approximately 3-, 6-, and 13-fold, respectively. This order of binding corresponds to the electronegativity of these groups (i.e. amino < iodo < chloro) and the extent of ionization (i.e. 9, 39, and 100% for uracil (**1**), 6-aminouracil (**36**), and 6-chlorouracil (**33**), respectively [18]). However, in contrast to 5-fluorouracil (**16**), 6-chlorouracil (**33**) ionizes predominantly at *N*1 [23], leaving the *N*3 proton intact. These results support our contention that increased ionization of a compound, at the appropriate location on the pyrimidine ring, enhances binding to *T. gondii* OPRTase.

Negatively charged exocyclic substituents at the C6 clearly increase binding to OPRTase (Table 1), suggesting an interaction with positively charged amino acids at the catalytic site. The increase in binding observed for these substituents was in the following approximate order: carboxyl > carboxy methyl ester > hydroxyl > methylsulfonyl = carboxymethyl. Not surprisingly, the addition of a carboxyl group to uracil (**1**) (orotic acid, **54**) markedly increased binding by about 87-fold. However, even larger increases (129- and 745-fold, respectively) were seen when a carboxyl group was substituted at the 6-position of 5-azauracil (**15**) or 1-deazauracil (**2**) to make 5-azaorotate (**59**) and 1-deazaorotate (**55**), respectively. The same trend, albeit to a lesser degree, was seen for several other compounds, which differed only in a 6-position carboxyl group (e.g. 5-fluorouracil, **16**, and 5-fluoroorotic acid, **61**; 5-bromouracil, **18**, and 5-bromoorotic acid, **62**; 5-iodouracil, **19**, and 5-iodoorotic acid, **63**; and 2-thiouracil, **8**, and 2-thioorotic acid, **57**). Substitution of a hydroxyl group at the 6-position had similar, although less dramatic, effects on binding to OPRTase (Table 1). The compounds resulting from the addition of a hydroxyl group to uracil (**1**), 2-thiouracil (**8**), and 5-azauracil (**15**) (i.e. barbituric acid, **46**; 2-thiobarbituric acid, **48**; and 5-azabarbituric acid, **50**) all bound better to OPRTase than their parent compounds. It should be noted, however, that substitution of either a carboxy or hydroxyl group at the 6-position of 5-nitrouracil (**26**) (i.e. 5-nitroorotic acid, **64**, and 5-nitrobarbituric acid, **53**, respectively) decreased or abolished binding. Whether this decrease is due to a change in ionization or to some interaction between the charged groups at the 5- and 6-positions is unknown. Nevertheless, the present data strongly indicate that a negatively charged exocyclic substituent at the 6-position promoted binding to *T. gondii* OPRTase.

Substitution at C6 with either a methyl (6-methyluracil, **39**) or benzyl group (6-benzyluracil, **40**) abolished binding to OPRTase. The lack of binding of these compounds was probably due to the hydrophobicity of the methyl and benzyl groups, which would prohibit an interaction with charged amino acids in the catalytic site of the enzyme, and (in the case of a benzyl group) steric hindrance.

other substitutions. 4,6-Dihydroxypyrimidine (**44**) bound approximately the same as uracil (**1**). This compound predominates in the diketo form and is highly ionized (100%) under our assay conditions [18]; however, its ionization mechanism is unknown. It should be noted that this compound can be rotated 180° about its *N*1-C4 axis and thus be considered as "5-aza-3-deazauracil." It is unclear whether this compound binds to OPRTase in an orientation where it has C2 and C4 oxo groups but lacks the *N*3 proton, or where it has an *N*3 proton and C4 and C6 oxo groups, but lacks a C2 oxo group. Both DL- (**66**) and L-dihydroorotic acid (**67**) bound poorly to OPRTase, suggesting that the C5-C6 double bond was required for binding.

Design of Ligands of T. gondii *OPRTase*

Table 2 summarizes the structure–activity relationship for the binding of ligands to *T. gondii* OPRTase, as determined from the results in Table 1. Based on these findings, several compounds that were not available can be proposed as potential ligands of *T. gondii* OPRTase. For example, 1-deazabarbituric acid (i.e. 2,4,6-trihydroxypyridine) would be predicted to be a good ligand for OPRTase, based on the binding of barbituric acid (**46**) and the increased binding of 1-deazaorotate (**55**) relative to orotate (**54**). The improved binding of 6-chlorouracil (**33**) and 6-iodouracil (**34**), as compared with uracil (**1**), suggests that uracil analogues with other electronegative groups at the 6-position (e.g. fluoro, bromo, or nitro) may be good ligands of OPRTase. It should be noted, however, that these compounds may not be stable enough to be useful as ligands. Finally, it would be useful to test certain halogen-substituted compounds to complete the structure–activity analysis. These would include 5-"halogen"barbituric acids, 1-deaza-5-"halogen"orotic acids, and 5-chloroorotic acid (which were not available).

TABLE 2. Structure–activity relationship for the binding of ligands to *T. gondii* OPRTase

Position*	Substituent effect
*N*1	An endocyclic pyridine-type nitrogen not required for binding; replacement with endocyclic methine does not affect (e.g. 1-deazauracil, **2**) or enhances (e.g. 1-deazaorotic acid, **55**) binding (8-fold).
	Exocyclic hydrophobic substituents larger than hydrogen abolish binding, possibly due to substituents overlapping with the PRibPP binding site and/or steric hindrance. Exception: orotidine (**56**) binds to the enzyme (albeit 71-fold less than orotic acid), possibly due to a 6-position negative charge.
C2, C4	Exocyclic oxo groups at both positions strongly preferred for binding; elimination of an oxo group (e.g. 4-hydroxypyrimidine, **6**; or 2-hydroxypyrimidine, **13**), or substitution with an amino group (e.g. 2-aminouracil, **7**; 4-aminouracil, **14**) at either position abolished binding. An exocyclic thio group at the 2-position (2-thiouracil, **8**; 2-thiobarbituric acid, **48**; and 2-thioorotic acid, **57**) decreases binding (3-, 2-, and 56-fold, respectively), while a thio group at both the 2- and 4-positions (e.g. 2,4-dithiouracil, **41**) abolishes binding.
*N*3	An endocyclic pyridine-type nitrogen required for binding; endocyclic substitutions markedly decrease (e.g. 3-deazauracil, **9**) or abolish (e.g. 3-oxauracil, **10**) binding.
	Exocyclic hydrophobic substituents larger than hydrogen (e.g. 3-methyluracil, **11**) abolish binding, possibly due to the lack of *N*3 hydrogen and/or steric hindrance.
	*N*3 proton believed to play a significant role in binding, probably due to hydrogen bonding with catalytic site; all of the best ligands evaluated in this study have an *N*3 proton (e.g. barbituric acid, **46**; orotic acid, **54**; or 1-deazaorotic acid, **55**).
C5	Endocyclic methine not required for binding; replacement with endocyclic pyridine type nitrogen (e.g. 5-azauracil, **15**; or 5-azaorotic acid, **59**) did not adversely affect binding.
	Compounds with exocyclic substituents similar in size to hydrogen strongly preferred for binding; exocylcic substituents larger than hydrogen, regardless of level of electronegativity, hydrophilicity, or hydrophobicity, (e.g. bromo, **18**; amino, **21**; or methyl, **28**) decrease or abolish binding, probably due to steric hindrance.
	Addition of a fluoro group to uracil (5-fluorouracil, **16**) or orotic acid (5-fluoroorotic acid, **61**) increased (1.7-fold) and decreased (4.1-fold) binding, respectively. Degree of binding may be due to pattern of pyrimidine ring ionization (i.e. ionization at *N*1 vs *N*3).
C6	Endocyclic methine not required for binding; replacement with endocyclic pyridine type nitrogen (e.g. 6-azauracil, **32**) did not adversely affect binding.
	Electron-withdrawing exocyclic substituents that increase pyrimidine ring ionization (e.g. chloro, **33**; or iodo, **34**) increase binding, possibly due to increased negative charge on exocyclic C2 and C4 groups, which may result in hydrogen bonding with the catalytic site.
	Negatively charged exocyclic substituents markedly increase binding (e.g. barbituric acid, **46**; orotic acid, **54**), probably due to an interaction with positively charged amino acids at the catalytic site.

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

Comparison of T. gondii *and Mammalian OPRTase*

A comparison of the structure–activity relationships for *T. gondii* OPRTase (present study) versus mammalian (mouse liver) OPRTase [19] lent some insight into the similarities and differences of the active site of these two enzymes. Similarities between the enzymes included diminished binding by compounds with any exocyclic substituent at the *N*1, C2, *N*3 and/or C4 positions; diminished binding by analogues with hydrophobic exocyclic substituents at C5 or C6 (e.g. 5-methyluracil, **28**; 6-methyluracil, **39**); and increased binding by compounds with negatively charged exocyclic substituents at C6 (e.g. carboxyl, **54**; hydroxyl, **46**). Differences between the enzymes included: improved binding of analogues substituted at endocyclic *N*3 nitrogen (e.g. 3-deazauracil, **9**; 3-oxauracil, **10**) to mammalian but not *T. gondii* OPRTase; a marked increase in binding by analogues with electron-withdrawing exocyclic substituents at C5 (e.g. 5-fluorouracil, **16**) to mammalian but not *T. gondii* OPRTase; and a marked increase in binding by analogues with electron-withdrawing exocyclic substituents at C6 (e.g. 6-chlorouracil, **33**) to *T. gondii* but not mammalian OPRTase.

Compounds that bind selectively to the parasite enzyme versus the mammalian enzyme may be exploited as potential chemotherapeutic agents. With the exception of 1-deazaorotic acid (**55**), all relevant compounds tested in the present study also were tested as ligands of mammalian OPRTase by Niedzwicki *et al.* [19]. Therefore, we estimated the apparent K_i value for 1-deazaorotic acid (**55**) for mammalian (mouse liver) OPRTase (using the same methodology and enzyme source used by Niedzwicki *et al.* [19]) and found it to be 34 ± 11 μM. Due to the differences in methodologies between the *T. gondii* and mammalian studies, it is difficult to

compare the apparent K_i values directly for the analogues tested against these two enzymes. However, we were able to identify four compounds, which had apparent K_i values that were approximately 50 μM or less for the *T. gondii* enzyme and were at least 10-fold lower than the corresponding values for the mammalian enzyme. These compounds (6-chlorouracil, **33**; 5-azaorotic acid, **59**; 1-deazaorotic acid, **55**; 6-iodouracil, **34**) have apparent K_i values for the *T. gondii* enzyme that are 14-, 21-, 72-, and 102-fold less, respectively, than for the mammalian enzyme [19 and present study]. Thus, these compounds may bind selectively to the parasite enzyme, making them good candidates for future testing as potential anti-toxoplasmic agents *in vivo*.

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