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STRUCTURE–ACTIVITY RELATIONSHIPS FOR THE BINDING OF LIGANDS TO XANTHINE OR GUANINE PHOSPHORIBOSYLTRANSFERASE FROM *TOXOPLASMA GONDII*

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Abstract—Preliminary characterization of *Toxoplasma gondii* phosphoribosyltransferase activity towards purine nucleobases indicates that there are at least two enzymes present in these parasites. One enzyme uses hypoxanthine, guanine, and xanthine as substrates, while a second enzyme uses only adenine. Furthermore, competition experiments using the four possible substrates suggest that there may be a third enzyme that uses xanthine. Therefore, sixty-eight purine analogues and thirteen related derivatives were evaluated as ligands of *T. gondii* phosphoribosyltransferase, using xanthine or guanine as substrates, by examining their ability to inhibit these reactions *in vitro*. Inhibition was quantified by determining apparent K_i values for compounds that inhibited these activities by greater than 10% at a concentration of 0.9 mM. On the basis of these data, a structure-activity relationship for the binding of ligands to these enzymes was formulated using hypoxanthine (6-oxopurine) as a reference compound. It was concluded that the following structural features of purine analogues are required or strongly preferred for binding to both enzymes: (1) a pyrrole-type nitrogen (lactam form) at the 1-position; (2) a methine (=CH—), a pyridine type nitrogen (=N—), or an exocyclic amino or oxo group at the 2-position; (3) no exocyclic substituents at the 3-position; (4) an exocyclic oxo or thio group in the *one* or *thione* tautomeric form at the 6-position; (5) a pyridine-type nitrogen (=N—) or a methine group at the 7-position; (6) a methine group at the 8-position; (7) a pyrrole-type nitrogen or a carbon at the 9-position; and (8) no exocyclic substituents at the 9-position. These findings provide the basis for the rational design of additional ligands of hypoxanthine, guanine, and xanthine phosphoribosyltransferase activities in *T. gondii*.

Key words: toxoplasma; purines; phosphoribosyltransferase; metabolism; chemotherapy

Toxoplasma gondii infections have become one of the most common causes of morbidity and mortality in individuals suffering from AIDS. The primary pathology observed is toxoplasmic encephalitis, which is reported to occur in 3–40% of these patients [1, 2]. Congenital toxoplasmosis is also a significant health problem, the incidence of which is as high as 1 in 1000 live births, with 50% of infected women giving birth to infected infants in the absence of treatment [2]. Congenital toxoplasmosis can lead to severe eye and brain damage and even death. Few drugs are available for the treatment of toxoplasmosis, and those therapies that do exist suffer from severe side-effects and the development of resistance [1, 3]. Therefore, the search for more efficacious and less toxic therapies for the treatment of toxoplasmosis needs to be continued.

One potential target for chemotherapeutic intervention against *T. gondii* is nucleotide metabolism. These parasites replicate rapidly and require large amounts of purines and pyrimidines for the synthesis of nucleic acids and other macromolecules. Indeed, the current standard treatment for *T. gondii* infections (antifolates and sulfonamides) uses this approach by blocking folate metabolism, thereby depriving the parasite of the necessary cofactors required for nucleotide biosynthesis [1, 3]. Furthermore, in contrast to their mammalian host, *T. gondii* are incapable of *de novo* purine biosynthesis and are totally dependent on the salvage pathways for their purine requirements [4, 5]. Therefore, these parasites can be selectively deprived of vital purines by blocking or interfering with their purine salvage pathways. The information available on the biochemistry and metabolism of natural purines and their analogues in *T. gondii* [4–10] suggests that these parasites differ from their host in various aspects of purine salvage metabolism. Such differences offer potential targets for the chemotherapy of toxoplasmosis.

One of the significant enzymatic reactions in the purine salvage pathways in *T. gondii* is the conversion of purine nucleobases, e.g. adenine, guanine, hypoxanthine, and xanthine, to their respective nucleoside 5′-monophosphates by phosphoribosyltransferase activities [4–6, 9]. These activities provide excellent targets for chemotherapeutic intervention because they play a crucial role in the salvage of purines in *T. gondii*. In addition to the nucleobases, inosine and guanosine are also incorporated into the nucleotide pool via this activity, since *T. gondii* lack any detectable nucleoside kinase activity towards these nucleosides [5]. Thus, inosine and guanosine are first cleaved by purine nucleoside phosphorylase (EC 2.4.2.1) to hypoxanthine and guanine, respectively, and then are converted to nucleotides by the action of phosphoribosyltransferases. Hence, purine analogues that inhibit *T. gondii* phosphoribosyltransferase activity would virtually block the salvage of most purines in this parasite. It is also important to emphasize that, unlike

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hypoxanthine-guanine phosphoribosyltransferases (EC 2.4.2.8) from mammalian hosts, the activity from *T. gondii* efficiently utilizes xanthine as substrate in addition to hypoxanthine and guanine [5]. In fact, it was reported that XPRTase¶ is the highest purine phosphoribosyltransferase activity in *T. gondii* [5]. The human enzyme shows only trace levels of activity with xanthine ([11–13], and unpublished results). This virtual lack of mammalian phosphoribosyltransferase activity towards xanthine constitutes a distinct difference between the host and parasite enzymes and suggests differences between the active sites of the two enzymes. Furthermore, it appears that the conversion of xanthine to its nucleotide XMP is its only metabolic fate since, unlike its host, *T. gondii* have no xanthine oxidase (EC 1.1.3.22) activity towards xanthine or hypoxanthine [5]. Therefore, XPRTase is a unique enzyme activity that plays a crucial role in purine salvage in *T. gondii*. The importance and distinction of phosphoribosyltransferase activities in *T. gondii* could be exploited in the development of antitoxoplasmic drugs. In particular, xanthine analogues that are activated to nucleotides by the phosphoribosyltransferase activity should be selectively toxic to the parasites.

In the present study, eighty-one compounds (eighty nitrogen heterocycles and one aromatic nitrile) were evaluated as ligands of *T. gondii* phosphoribosyltransferase, using xanthine (XPRTase) or guanine (GPRTase) as a substrate, by examining their ability to inhibit these activities *in vitro*. The use of both xanthine and guanine was necessary because our preliminary characterization of purine phosphoribosyltransferase activities in extracts of *T. gondii* suggested that there may be a separate enzyme that specifically uses xanthine as a substrate. Furthermore, the use of both xanthine and guanine may help in delineating whether one or two phosphoribosyltransferase(s) utilize(s) these two purines. Inhibition was quantified by determining apparent K_i values for compounds that inhibited enzyme activities by greater than 10% at a concentration of 0.9 mM. On the basis of these data, structure–activity relationships for the binding of purine nucleobase analogues to these activities were formulated, in order to identify and/or design analogues that may be specific inhibitors or substrates of *T. gondii* XPRTase or GPRTase.

MATERIALS AND METHODS

Chemicals and supplies

The source of the compounds screened as inhibitors are indicated in Table 3 by the following abbreviations: ALD, Aldrich Chemical Co., Inc., Milwaukee, WI; BWC, Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC; CDC, Chemical Dynamics Corp., South Plainfield, NJ; FLU, Fluka Chemical Co., Ronkonkoma, NY; NBC, Nutritional Biochemicals Corp., Cleveland, OH; RPP, Dr. Raymond P. Panzica, University of Rhode Island, Kingston, RI; RSK, Dr. Robert S. Klein, Montefiore Medical Center, Bronx, NY; SHC, Dr. Shih-Hsi Chu, Brown University, Providence, RI; and SIG, Sigma Chemical Co., St. Louis, MO. [8-^{14}C]Adenine (55 Ci/mol), [8-^{14}C]guanine (55 Ci/mol), [8-^{14}C]hypoxanthine (55 Ci/mol), and [8-^{14}C]xanthine (55 Ci/mol) were obtained from Moravek Biochemicals, Inc., Brea, CA; cellulose CEL 300 UV_{254} Polygram TLC 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 the Sigma Chemical Co.

Maintenance of T. gondii

Tachyzoites of the RH strain of *T. gondii* were propagated by intraperitoneal passage in female CD-1 mice (Charles River Laboratories, Wellington, MA) weighing 20–25 g. Mice were injected intraperitoneally with an inoculum of *T. gondii* (10^6 parasites) contained in 0.2 mL of sterile phosphate-buffered saline (pH 7.2) and killed after 2–3 days by inhalation of ether. The parasites were harvested from the peritoneal cavity by injecting and then aspirating 3–5 mL of phosphate-buffered saline (2–3 times). The peritoneal fluid was examined microscopically to determine the concentration of *T. gondii* and to ascertain the extent of contamination by host cells. Two-day transfers generally produced parasite preparations that contained very little contamination and had a purity of >97%.

Preparation of enzyme extracts

T. gondii suspension in phosphate-buffered saline was washed 2–3 times in 50 mM Tris-Cl, pH 7.4, at room temperature. Enzyme extracts were prepared by sonicating live parasites in 3 vol. of 50 mM Tris-Cl, pH 7.4, using a Fisher Sonic Dismembrator (model 300) on ice. The sonicate (enzyme extract) was used as the enzyme source.

Phosphoribosyltransferase assays

Phosphoribosyltransferase activity was measured by following the formation of [^{14}C]nucleoside 5′-monophosphate (and [^{14}C]nucleoside formed by phosphohydrolase activity) from [^{14}C]nucleobase and PRibPP. The standard reaction mixtures contained 50 mM Tris–Cl (pH 7.4), 10 μM [8-^{14}C]xanthine (55 Ci/mol) or 4 μM [8-^{14}C]guanine (55 Ci/mol), 4 mM PRibPP, 20 mM $MgCl_2$, 50 mM KCl, and 50 μL of enzyme extract (4–8 μg of protein) in a final volume of 100 μL. When analogues were being tested, various concentrations (0 to 0.9 mM) of the analogue were also included.

Reactions were incubated at 37° and terminated in boiling water bath for 2 min. Precipitated proteins were removed by centrifugation. A 10-μL aliquot of the resulting supernatant was spotted on cellulose TLC plates that were developed with 5% dibasic potassium phosphate. The average R_f values were as follows: hypoxanthine, 0.49; inosine, 0.65; IMP, 0.77; guanine, 0.14; guanosine, 0.57; GMP, 0.70; xanthine, 0.46; xanthosine, 0.61; XMP, 0.74; adenine, 0.33; adenosine, 0.46; and AMP, 0.67. The radioactivity was quantified using a Berthold TLC Linear Analyzer (Wallac Inc., Gaithersburg, MD). All assays were run under conditions in which velocity was linear with respect to time and amount of enzyme extract. Enzyme velocity was calculated by multiplying the fraction of nucleotide plus nucleoside formed from nucleobase by the amount of nucleobase in the assay divided by the incubation time.

¶Abbreviations: GPRTase, phosphoribosyltransferase activity with guanine; PRibPP, 5-phosphoribosyl-1-pyrophosphate; and XPRTase, phosphoribosyltransferase activity with xanthine.

Specific activity was estimated by dividing enzyme velocity by the amount of protein in the assay.

Determination of apparent K_m *values*

The assay conditions were the same as for the standard assay except for the substrate concentrations used. The range of substrate concentrations was 1–7 μM. Apparent V_{max} and K_m values were calculated using a computer program written by Dr. Sungman Cha (Brown University, Providence, RI) and fitted into IBM BASIC by Dr. Fardos N. M. Naguib. This program employs the Wilkinson–Cleland procedure [14, 15] for the estimation of V_{max} and K_m.

Determination and significance of apparent K_i *values*

Apparent K_i values were estimated from Dixon plots of the data [1/v vs (I)], using a computer program that employs least-squares fitting according to the general principles of Cleland [15]. This program was developed by Drs. S. Cha and F. N. M. Naguib. If a compound is a competitive inhibitor with respect to the substrate, apparent K_i values are related to K_i values by the following equation [16]: apparent $K_i = K_i$ (1 + [S]//K_m). In the present study, the concentration of the purine nucleobase (10 and 4 μM for xanthine and guanine, respectively) was about 3-fold greater than its approximate apparent K_m value (Table 1) and the concentration of PRibPP (4 mM) was at a saturating concentration (results not shown). Thus, the apparent K_i value determined for a competitive inhibitor would be about 4-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 [17] using the Bio-Rad Laboratories protein assay kit and bovine serum albumin as a standard.

RESULTS

Characterization of T. gondii *phosphoribosyltransferases*

Determination of kinetic parameters. Table 1 presents the apparent K_m and V_{max} values for *T. gondii* phosphoribosyltransferase activity using hypoxanthine, guanine, xanthine, or adenine as substrates. In addition, the efficiencies of these activities (i.e. V_{max}/K_m) are also shown in Table 1 and were found to be in the order hypoxanthine > guanine > xanthine > adenine. This ranking differs from the previously reported [5] order of specific activities, which was xanthine > hypoxanthine > guanine > adenine. This discrepancy does not appear to be due to the assay conditions employed in the present study, as similar results were obtained when assays were conducted using the previously reported conditions [5].

Determination of pH optima. The pH optima for *T. gondii* phosphoribosyltransferase activity using hypoxanthine, guanine, xanthine, or adenine as substrates were found to be 7.5, 7.5, 7.8, and 8.0–9.5, respectively. Thus, the order of efficiencies for these substrates (Table 1) may at least partially reflect the difference between the pH employed (i.e. 7.4) and the pH optima for the different substrates.

Competition studies. Competition studies between the four substrates for *T. gondii* phosphoribosyltransferase activity were conducted in order to ascertain how many separate enzyme activities are present in these parasites. As can be seen in Table 2, adenine did not inhibit either GPRTase or XPRTase. Both hypoxanthine and xanthine were competitive inhibitors of GPRTase, whereas hypoxanthine was a noncompetitive inhibitor of XPRTase and guanine was a competitive inhibitor. These results indicate that at least two phosphoribosyltransferases are present in *T. gondii*. One enzyme uses hypoxanthine, guanine, and xanthine as substrates, while the other enzyme uses only adenine. However, the noncompetitive inhibition demonstrated by hypoxanthine towards XPRTase suggests that there may be a third enzyme that uses xanthine.

Evaluation of nucleobase analogues as ligands of T. gondii *XPRTase and GPRTase*

Eighty-one compounds, mostly purine analogues, were evaluated as ligands of *T. gondii* XPRTase and GPRTase by examining their ability to inhibit these activities *in vitro*. The purine nucleobase analogues that were screened included those having ring modifications (e.g. aza and deaza analogues) and/or exocyclic substitutions at various positions. Inhibition was quantified by determining apparent K_i values for those compounds that inhibited XPRTase or GPRTase by greater than 10% at a concentration of 0.9 mM. The mean and range of the apparent K_i values for these compounds, determined from at least three separate estimations of the apparent K_i, are shown in Table 3.

DISCUSSION

Tautomerism of hypoxanthine

Nitrogen heterocycles including purines experience tautomerism, a process that involves interconversion of

Table 1. Kinetic parameters for *T. gondii* phosphoribosyltransferases

Substrate	Apparent K_m* (μM)	Apparent V_{max}* (nmol/min/mg protein)	Efficiency (V_{max}/K_m)
Hypoxanthine	1.2 ± 0.1	0.94 ± 0.01	0.78
Guanine	1.3 ± 0.4	0.72 ± 0.07	0.55
Xanthine	3.0 ± 0.4	1.1 ± 0.1	0.37
Adenine	2.9 ± 0.8	0.099 ± 0.007	0.03

* Values are means ± SD from at least three estimations.

Table 2. Inhibition of *T. gondii* XPRTase and GPRTase by alternative substrates

Substrate	Inhibitor	K_{is}* (μM)	Type of inhibition
Guanine	Hypoxanthine	0.4 ± 0.1	Competitive
	Xanthine	3.7 ± 1.2	Competitive
	Adenine		None
Xanthine	Hypoxanthine	0.3 ± 0.1	Noncompetitive
	Guanine	1.2 ± 0.1	Competitive
	Adenine		None

* Values are means ± SD from at least three estimations.

Table 3. Apparent K_i values for inhibition of *T. gondii* XPRTase and GPRTase

Compound*	Source	Apparent K_i† (μM) XPRTase	GPRTase
1 Hypoxanthine (6-oxopurine)	SIG	3.5 ± 0.3	1.1 ± 0.1
2-Position substitutions			
2 2-Aminohypoxanthine (guanine)	SIG	12 ± 2	10 ± 2
3 2-Methylaminohypoxanthine (N^2-methylguanine)	SIG	‡	‡
3 2-Oxohypoxanthine (xanthine)	SIG	14 ± 2	16 ± 4
5 2-Thiohypoxanthine (2-thioxanthine or 6-oxo-2-thiopurine)	SIG	68 ± 18	66 ± 11
6-Position substitutions			
6 Purine	CDC	‡	‡
7 6-Aminopurine (adenine)	SIG	‡	‡
8 6-Methylaminopurine (N^6-methyladenine)	SIG	970 ± 170	770 ± 280
9 6-Benzylaminopurine (N^6-benzyladenine)	SIG	1100 ± 300	1400 ± 600
10 6-Methoxypurine	CDC	380 ± 90	400 ± 80
11 6-Thiopurine (purine-6-thione)	CDC	6 ± 1	15 ± 1
12 6-Chloropurine	CDC	100 ± 30	48 ± 3
13 6-Iodopurine	CDC	‡	‡
14 6-Methylpurine	CDC	‡	1100 ± 500
8-Position substitutions			
15 8-Azahypoxanthine (8-aza-6-oxopurine)	CDC	2000 ± 500	610 ± 190
16 8-Oxohypoxanthine (6,8-dioxopurine)	CDC	4100 ± 1000	‡
17 8-Thiohypoxanthine (6-oxopurine-8-thione)	CDC	420 ± 100	500 ± 150
1,2-Position substitutions			
18 2-Amino-1-methylhypoxanthine (1-methylguanine)	SIG	40 ± 5	150 ± 30
19 2-Oxo-1-methylhypoxanthine (1-methylxanthine)	FLU	1400 ± 400	3700 ± 400
2,6-Position substitutions			
20 2-Aminopurine	CDC	67 ± 32	280 ± 96
21 2-Oxopurine (2-hydroxypurine)	SIG	570 ± 120	430 ± 110
22 2,6-Diaminopurine (2-aminoadenine)	CDC	880 ± 280	460 ± 210
23 6-Amino-2-oxopurine (isoguanine)	NBC	53 ± 15	220 ± 60
24 6-Amino-2-chloropurine (2-chloroadenine)	CDC	2200 ± 900	630 ± 180
25 6-Amino-2-methylpurine (2-methyladenine)	SIG	370 ± 110	2500 ± 800
26 2-Aminopurine-6-thione (6-thioguanine)	SIG	2″ ± 12	6 ± 0.3
27 2,6-Dithiopurine (dithioxanthine)	CDC	420 ± 80	300 ± 30
28 2-Oxo-6-thiopurine (6-thioxanthine)	SIG	246 ± 82	318 ± 128
29 2-Amino-6-chloropurine	CDC	230 ± 50	890 ± 170
30 2,6-Dichloropurine	CDC	410 ± 80	3000 ± 1600
2,7-Position substitutions			
31 2-Amino-7-deazahypoxanthine (7-deazaguanine)	SIG	26 ± 5	93 ± 6
32 2-Amino-7-methylhypoxanthine (7-methylguanine)	SIG	3100 ± 1500	3200 ± 2000
33 2-Oxo-7-methylhypoxanthine (7-methylxanthine)	CDC	1900 ± 400	5700 ± 2300
2,8-Position substitutions			
34 8-Aza-2-aminohypoxanthine (8-azaguanine)	CDC	2600 ± 1000	450 ± 90
35 8-Bromo-2-aminohypoxanthine (8-bromoguanine)	SIG	540 ± 120	1600 ± 400
36 8-Aza-2-oxohypoxanthine (8-azaxanthine)	CDC	‡	‡
6,8-Position substitutions			
37 8-Aza-6-aminopurine (8-azaadenine)	SIG	3500 ± 1400	1800 ± 800
38 6-Amino-8-bromopurine (8-bromoadenine)	CDC	750 ± 1000	630 ± 140
6,9-Position substitutions			
39 6-Amino-9-ethylpurine (9-ethyladenine)	CDC	‡	1000 ± 400
40 6-Amino-9-cyclohexylpurine (9-cyclohexyladenine)	CDC	‡	3200 ± 800
Other di-substitutions			
41 6-Amino-1-methylpurine (1-methyladenine)	CDC	310 ± 90	5900 ± 3000
42 2-Oxohypoxanthine-N^3-oxide (xanthine-N^3-oxide)	SIG	1300 ± 300	5500 ± 1200
43 2-Oxo-3-methylhypoxanthine (3-methylxanthine)	FLU	‡	‡
44 2-Amino-9-deazahypoxanthine (9-deazaguanine)	RSK	14 ± 1	15 ± 1
45 2-Amino-9-methylhypoxanthine (9-methylguanine)	CDC	230 ± 50	610 ± 150
46 2-Aza-3-deazahypoxanthine ·	RPP	22 ± 4	25 ± 7
47 8-Aza-7-deazahypoxanthine (allopurinol)	SIG	35 ± 6	130 ± 20

Table 3. (*Continued*)

Compound*	Source	Apparent K_i† (μM) XPRTase	Apparent K_i† (μM) GPRTase
Multiple substitutions			
48 2-Amino-1-methylpurine	SIG	290 ± 90	730 ± 180
49 2,6-Dioxo-3-isobutyl-1-methylpurine	SIG	1500 ± 600	‡
50 6-Amino-7-deazapurine (7-deazaadenine)	BWC	1700 ± 500	5800 ± 900
51 8-Aza-6-oxo-1,3-dimethylpurine	SHC	‡	‡
52 1,2,4-Triazolo(1,5-*a*)pyrimidine(1-deaza-5-azapurine)	ALD	‡	‡
53 8-Aza-2,6-diaminopurine (8-aza-2-aminoadenine)	SIG	850 ± 230	1300 ± 400
54 8-Aza-7-deaza-6-aminopurine (8-aza-7-deazaadenine)	CDC	81 ± 52	‡
55 8-Aza-7-deaza-6-thiopurine	BWC	880 ± 330	970 ± 120
56 8-Aza-1,3-dideazapurine (benzotriazole)	CDC	5200 ± 2300	‡
57 8-Aza-1-nitro-1,3-dideazapurine (5-nitrobenzotriazole)	ALD	390 ± 430	‡
58 2,6-Dioxo-7-(*b*-hydroxypropyl)-1,3-dimethylpurine	SIG	1900 ± 1200	‡
59 2,6-Dioxo-1,3,7-trimethylpurine (caffeine)	SIG	1000 ± 400	840 ± 280
60 2,6-Dioxo-1,3,9-trimethylpurine (isocaffeine)	FLU	5300 ± 1300	6100 ± 2400
61 3-Methyl-2,6,8-trioxopurine	FLU	‡	‡
62 1,3-Dimethyl-2,6,8-trioxopurine	FLU	‡	‡
63 2,8-Diaza-3-deazahypoxanthine	RPP	‡	‡
64 6-Amino-2,8-diaza-3-deazapurine	RPP	437 ± 109	160 ± 31
65 2-Aza-6-amino-3-deazapurine (2-aza-3-deazadenine)	RPP	231 ± 35	135 ± 19
66 2-Aza-6-thio-3-deazapurine	RPP	30 ± 6	14 ± 3
67 2-Aza-6-methylthio-3-deazapurine	RPP	‡	‡
68 1,3,7-Trideazahypoxanthine (4-hydroxyindole)	ALD	‡	‡
Other substitutions			
69 Benzonitrile	CDC	870 ± 290	580 ± 170
70 1,3-Dimethylpteridine	SHC	‡	‡
71 6-Hydroxymethylpterin	SIG	3400 ± 1800	6600 ± 4500
72 Cytosine (4-amino-2-oxopyrimidine)	SIG	286 ± 60	476 ± 90
73 5-Fluorocytosine	CDC	657 ± 180	431 ± 100
74 Isocytosine (2-amino-4-oxopyrimidine)	NBC	1060 ± 270	616 ± 200
75 Uracil (2,4-dioxopyrimidine)	SIG	808 ± 20	699 ± 140
76 4-Oxopyrimidine (4-hydroxypyrimidine)	CDC	379 ± 100	445 ± 130
77 Imidazole	SIG	‡	‡
78 2-Aminoimidazole	RPP	‡	‡
79 5(4)-Amino-4-imidazolecarboxamide (AICA)	RPP	7.9 ± 1.3	9.0 ± 1.2
80 Methyl 5(4)-hydroxymethylimidazole-4(5)carboxylate	RPP	‡	‡ ‡
81 4(5)-Nitro-5(4)-sulfamoylimidazole	RPP	‡	

* Compounds are listed mainly as analogues of hypoxanthine (**1**) or purine (**6**) with alternative names in parentheses. Chemical names of selected analogues are as follows (compound numbers in parentheses): 8-azahypoxanthine (**15**), 7-oxo-*v*-triazolo(4,5-*d*)pyrimidine; 7-deazaguanine (**31**), 2-amino-4-oxopyrrolo[2,3-*d*])pyrimidine; 8-aza-7-deazahypoxanthine (**47**), 4-oxopyrazolo[3,4-*d*]pyrimidine; 8-aza-7-deazaadenine (**54**), 4-aminopyrazolo[3,4-*d*]pyrimidine; 2,8-diaza-3-deazahypoxanthine (**63**), *v*-triazolo[**4,5**-*d*]pyridazine-4(5*H*)-one; 2-aza-6-thio-3-deazapurine (**66**), imidazole[4,5-*d*]pyridazine-4(5*H*)-thione.

† Apparent K_i values (means ± SD) were obtained from at least three separate estimations of the apparent K_i.

‡ Less than 10% inhibition at a concentration of 0.9 mM.

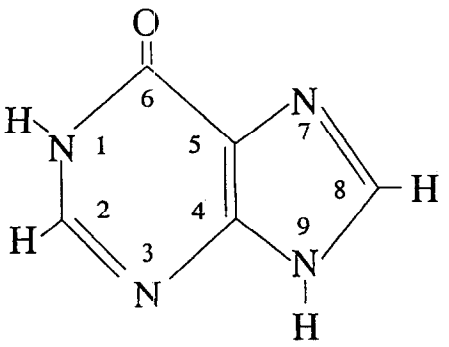

Fig. 1. Structure of the reference compound hypoxanthine with the numbering system for the purine ring.

structural isomers via migration of a proton: H-A-B = X ↔ A = B-X-H [18]. A particular tautomeric form may play a pivotal role in how certain nitrogen heterocycles may bind to biomolecules such as the active sites of enzymes, hence the importance of delineating the tautomerism of purines. In the following discussion, this important physicochemical characteristic will be taken into account in an effort to explain the findings of the present study.

As can be seen in Table 3, hypoxanthine (6-oxopurine, **1**) was the best ligand for both XPRTase and GPRTase and will be used as a reference compound throughout this discussion. Hypoxanthine exists predominately (>99%) as the *N*(1)H tautomer (lactam form, *C*(6)=O) in solution [19] (see Fig. 1). Two types of tautomerism can occur simultaneously in hypoxanthine (**1**), prototropic tautomerism in the imidazole moiety and lactam-lactim tautomerism in the pyrimidine portion. An

early study [20], which used UV and ^{1}H-NMR data, suggested that the *N*(1)H-*N*(9)H tautomer (shown in Fig. 1) predominates in a dilute aqueous solution, whereas a more recent study [19], based on ^{13}C carbon chemical shifts, indicated that the *N*(1)H-*N*(7)H tautomer is the predominant (*ca.* 58%) form. It should be noted that guanine (**2**) and xanthine (**4**) were also found to be two of the best ligands for *T. gondii* XPRTase and GPRTase (Table 3). Like hypoxanthine (**1**), both guanine (**2**) and xanthine (**4**) exist as their *N*(1)H tautomers with a keto group at *C*6 [21]. With respect to their *N*(1)H-*N*(7)H and *N*(1)H-*N*(9)H tautomeric populations, data suggest that guanine (**2**) exists as a mixture of these two tautomers [21] with the *N*(1)H-*N*(7)H form slightly more favorable [22]. On the other hand, xanthine (**4**) exists essentially as the *N*(7)H tautomer in aqueous solution [23]. It should be noted, however, that in the active site of an enzyme, the substrate may assume a high energy tautomeric form due to the influence of the enzyme *per se*.

Binding of ligands to XPRTase and GPRTase from T. gondii

The compounds screened in the present study are listed in Table 3 as analogues of hypoxanthine, and the results will be discussed primarily with respect to the type of substituents on hypoxanthine (Fig. 1) and how it affects binding to *T. gondii* XPRTase or GPRTase. However, due to the limited number of mono-substituted purines tested in the present study, the structure–activity relationships for the binding of ligands to these enzymatic activities were developed by taking into consideration a variety of mono-, di and tri-substituted compounds and their combined effect on enzyme activity. To simplify the discussion, the purine numbering system (Fig. 1) will be used, and compounds will be referred to as analogues of hypoxanthine or purine and cited by compound number (bold type numerals in parentheses).

1-*Position substitutions.* A pyrrole-type nitrogen (*N*H) appears to be strongly preferred or required for binding, as the presence of an exocyclic methyl group at *N*1 decreased binding to both XPRTase and GPRTase. For example, 1-methylguanine (**18**) bound to XPRTase and GPRTase 3.3- and 15-fold less than guanine (**2**), respectively, while 1-methylxanthine (**19**) bound to these enzymes 100- and 230-fold less than xanthine (**4**), respectively. The decreased binding of these compounds may be due to the absence of hydrogen bonding between the exocyclic proton at the 1-position as well as steric hindrance at the catalytic sites of the enzymes. The basis for the very poor binding of 1-methylxanthine (**19**) to these enzymes, as compared with 1-methylguanine (**18**), is unclear.

2-*Position substitutions.* Exocyclic substitutions on the 2-position of purine (**6**) generally increased binding. Substitution of an amino group on purine (2-aminopurine, **20**) enhanced binding to both XPRTase and GPRTase, while substitution of an oxo group on adenine (isoguanine, **23**) resulted in even better binding. The effect of this type of substitution was more pronounced for XPRTase than GPRTase. 1-Methylguanine (**18**), 2-aminopurine (**20**), isoguanine (**23**), 6-thioguanine (**26**), 7-deazaguanine (**31**), and 9-deazaguanine (**44**) bound well to XPRTase, whereas only 6-thioguanine (**26**) 7-deazaguanine (**31**) and 9-deazaguanine (**44**) bound to GPRTase. Of the six compounds, binding was enhanced when an oxo (*C*(6)=O) or thioxo (*C*(6)=S) group resided at *C*6. 2-Thiohypoxanthine (**5**) also exhibited moderate binding to both enzymes. Therefore, it appears that both XPRTase and GPRTase can accommodate substituents at *C*2, or even a replacement of *C*2 by a pyridine nitrogen (2-aza-3-deazahypoxanthine, **46**) that can participate in hydrogen bonding. Such bonding at the *C*2 position can occur with either an unshared electron pair (=$\ddot{N}$—, *C*=$\ddot{X}$:, or *C*—$\ddot{N}H_2$) or with a hydrogen as in the case of an amino group. In addition to the ability of such substituents at *C*2 to form hydrogen bonds, their relative size may also play a role in how they are accommodated by the active site of the enzyme. For example, the apparent K_i values for compounds with exocyclic amino (**2**), oxo (**4**), and thioxo (**5**) groups increased with the increase in the size of the substituent at *C*2. In fact, a methylamino (**3**) group at *C*2 completely abolished binding. A similar trend was observed for 6-thioguanine (**26**), dithioxanthine (**27**), and 2-amino-6-chloropurine (**29**) where the apparent K_i values were in the order 2-thioxo (**27**) > 2-amino (**26**), and 2-chloro (**30**) > 2-amino (**29**) for exocyclic substitutions at the 2-position of 6-thiopurine (**11**) or 6-chloropurine (**12**), respectively. At this time, it is unclear why AICA (**79**), which lacks the pyrimidine ring and an atom or substituent at the 2-position, binds extremely well to both XPRTase and GPRTase. These data suggest that the integrity of the pyrimidine portion of the purine ring is not an essential requirement for binding and that *N*1 and *C*6 positions [represented by the carboxamide function of AICA (**79**)], unlike *C*2, are not required components for binding.

3-*Position substitutions.* Exocyclic substitutions at *N*3 decreased or abolished binding to both XPRTase and GPRTase. The loss or decrease in binding appears to be a direct result of steric hinderance. For example, placing an *N*-oxide group at *N*3 of xanthine (i.e. xanthine-N^3-oxide), **42**) decreased binding to XPRTase and GPRTase by 93- and 340-fold, respectively, while substitution of a methyl group at this position (3-methylxanthine, **43**) abolished binding to both enzymes. Similarly, substitution of an isobutyl group to the 3-position of 1-methylxanthine (**19**) (i.e. 1-methyl-3-isobutylxanthine, **49**) also decreased (XPRTase) or abolished (GPRTase) binding. Our data also indicate that XPRTase and GPRTase accept only purines that have either an unsubstituted pyrrole-type nitrogen (NH) or a pyridine-type nitrogen (=$\ddot{N}$—) at the 3-position. The best examples are xanthine (**4**; NH at *N*3) and guanine (**2**; =$\ddot{N}$— at *N*3), which bound to both enzymes equally well. The imidazole 4(5)nitro-5(4)-sulfamoylimidazole (**81**), an analogue of AICA (**79**), did not bind to either XPRTase or GPRTase. The 4(5)nitro- on **81** has a similar spatial arrangement as the *N*3-oxide of xanthine-*N*3-oxide (**42**). As mentioned above, bulky substituents on the 3-position of purines drastically decrease binding to both enzymes.

6-*Position substitutions.* As mentioned above, hypoxanthine (**1**) is the best ligand for both GPRTase and XPRTase. This suggests that there is a strong interaction between the *C*6 oxo group and the catalytic sites of these enzymes, probably through hydrogen bonding. Surprisingly, substitution of oxo group at the 6-position of hypoxanthine (**1**), or the 2- and 6-positions of xanthine with a thio group, to yield 6-thiopurine (**11**) and 2,6-dithiopu-

rine (**27**), respectively, decreased binding to XPRTase and GPRTase relative to hypoxanthine (**1**) by 1.7- and 14-fold, and to xanthine (**4**) by 30- and 19-fold, respectively. This may reflect the greater percentage of the thiol tautomeric form in which the hydrogen residues on the sulfur (*C*(6)-SH vs *C*(6)=S) [19] rather than on the *N*1 nitrogen. A similar trend is observed for 2-aza-3-deazahypoxanthine (**46**) and 2-aza-6-thio-3-deazapurine (**66**) with XPRTase, but not with GPRTase. To the best of our knowledge, no studies exist that examine the degree of lactim-lactam or thiol-thione tautomerism of these two purine analogues. The interchange of the *N*3 and *C*2 positions of purine undoubtedly has some effect on the overall percentage of the thiol or lactim tautomeric forms of these heterocycles. Such an effect may explain why the sulfur analogue (**66**) binds better to GPRTase. The percentage of the thiol tautomeric form, coupled with the increase in size from oxygen to sulfur, could definitely influence binding. That both enzymes are sensitive to the size of the substituents was also demonstrated by the binding of 6-chloropurine (**12**) and 6-iodopurine (**13**). 6-Chloropurine (**12**) bound to both enzymes, although not as efficiently as 6-thiopurine (**11**), while 6-iodopurine (**13**) did not bind at all. Similar observations have been reported for the binding of 6-chlorouracil and 6-iodouracil to dihydrouracil dehydrogenase (EC 1.3.1.2), where the iodo substituent decreased binding by approximately 10-fold more than that seen for the chloro group [24]. Although the chloro group is more electronegative than an iodo group [25], the iodo (2.2 Å) is larger than the chloro (1.8 Å) [26]. Furthermore, the area of the active site of these enzymes, which accommodates the *C*6 substituents, appears to be more sensitive to size than the electronic nature of the substituent, i.e. electron-withdrawing vs electron-donating. This is evident from the gradual decrease in the binding of 6-methoxypurine (**10**), 6-methylaminopurine (**8**), 6-benzylaminopurine (**9**), and 6-methylpurine (**14**). It should be noted that all the latter purines lack a hydrogen at *N*1, which appears to be necessary for good binding to XPRTase and GPRTase.

7-Position substitutions. The nitrogen at position 7 may not be required for binding to either XPRTase or GPRTase, as replacing it with a methine group (e.g. 7-deazaguanine, **31**) decreased binding only slightly (2.2- and 9.3-fold, respectively). On the other hand, substitution of an exocyclic methyl group at the 7-position of guanine (i.e. 7-methylguanine, **32**) or xanthine (i.e. 7-methylxanthine, **33**) decreased binding to both enzyme activities by at least 136-fold. These data suggest that only a hydrogen atom or a nitrogen with an unshared pair of electrons can reside at the 7-position.

8-Position substitutions. A methine group (=*C*—) at the 8-position is required for binding to both XPRTase and GPRTase. Replacement of the 8-position methine of hypoxanthine (**1**), guanine (**2**) or xanthine (**4**) with a nitrogen, to yield 8-azahypoxanthine (**15**), 8-azaguanine (**34**), or 8-azaxanthine (**36**), respectively, significantly decreased (at least 400-fold) or abolished binding to both XPRTase and GPRTase. Prototropic tautomerism can occur in the triazole moiety of the 8-azapurines to give rise to three possible tautomeric forms, i.e. the *N*(7)H, *N*(8)H or *N*(9)H tautomers. Theoretical calculations have shown the *N*(8)H tautomer to be a high energy form and, therefore, much less likely to occur than the *N*(7)H or *N*(9)H tautomers [22]. As a result, the *N*8 nitrogen of 8-azapurines exists predominately as a "pyridine-type" nitrogen (=*N*̈—) that has an unshared pair of electrons that are not involved in the aromaticity of the heterocycle. Thus, the poor binding of the 8-azapurines studied may be due to the electronic influence of the 8-position nitrogen on hydrogen bonding at *N*7 or possibly charge repulsion within the catalytic sites of these enzymes. This hypothesis is supported by the fact that simultaneous replacement of an 8-position methine with a nitrogen and a 7-position nitrogen with a methine to yield 8-aza-7-deazahypoxanthine (**47**) or 8-aza-7-deaza-6-thiopurine (**55**) decreased binding by 10-fold. In addition, substitution at the *C*8 of hypoxanthine (**1**) with an oxo (8-oxohypoxanthine, **16**) or thio group (8-thiohypoxanthine, **17**), or a bromo group at *C*8 of guanine (**2**) to yield 8-bromoguanine (**35**), significantly decreased (at least 120-fold) or abolished binding to both XPRTase and GPRTase. Thus, the size of the substituents at *C*8 definitely affects the affinity of the analogue to the enzymes. Surprisingly, the 8-aza-7-deaza substitution to 6-aminopurine (adenine, **7**) to give 8-aza-7-deaza-6-aminopurine (8-aza-7-deazaadenine, **54**) greatly improved binding to both enzymes. These results suggest that altering the electron density of the entire heterocycle will indeed influence the ability of other functional groups on the purine to bind to the enzyme.

9-Position substitutions. The nitrogen at position 9 may not be required for binding to either XPRTase or GPRTase, since replacing it with a methine group (e.g. 9-deazaguanine, **44**) did not affect binding. On the other hand, substitution of an exocyclic methyl group at the 9-position of guanine (i.e. 9-methylguanine, **45**) decreased binding to both XPRTase and GPRTase (19- and 61-fold, respectively). The poor binding of 9-methylguanine (**45**) can be explained on the basis of the reaction mechanism of these enzymes. It is possible that PRibPP binds to the enzyme first, followed by the appropriate purine resulting in nucleotide formation. Thus, any exocyclic substituents at the 9-position larger than a hydrogen would preclude binding to these enzymes due to overlap with the PRibPP binding site.

Other substitutions. Since the structure of a purine can be considered as a fusion of an imidazole ring with a pyrimidine ring, several imidazoles and pyrimidines were examined as ligands for both XPRTase and GPRTase. The pyrimidines selected were similar in structure (i.e. the placement of functional groups were the same) to those found on the active purines. For example, 4-oxopyrimidine (**76**), uracil (**75**), isocytosine (**74**) and cytosine (**72**) can be considered to be analogues of hypoxanthine (**1**), xanthine (**4**), guanine (**2**) and isoguanine (**23**), respectively. The poor binding of these pyrimidine analogues to both XPRTase and GPRTase, relative to their purine counterparts, indicates that the imidazole moiety of purines is essential for the binding of ligands to these enzymes. The importance of the imidazole ring is further emphasized by the finding that compounds that have an incomplete pyrimidine ring (e.g. AICA, **79**) bind extremely well to both XPRTase and GPRTase. The lack of binding of methyl 5(4)-hydroxymethylimidazole-4(5)carboxylate (**80**) and 4(5)-nitro-5(4)-sulfamoylimidazole (**81**) when compared with AICA (**79**) could be attributed to the exocyclic substitution at the 1- and/or 3-position which, as discussed

Table 4. Structure-activity relationship for the binding of nucleobase ligands to *T. gondii* XPRTase and GPRTase

Position*	Substituent effect
1-Position	A pyrrole-type nitrogen strongly preferred or required for binding; substitution with an exocyclic methyl group (e.g. 1-methylguanine, **18**†; or 1-methylxanthine, **19**) decreases binding.
2-Position	Elimination of the endocyclic carbon does not affect binding (e.g. AICA, **79**). Exocyclic substitutions to purine (**6**) (e.g. oxo, **21**; or amino, **20**) generally increase binding; exocyclic substitutions to other compounds (e.g. hypoxanthine **1**) usually decrease (e.g. amino, **2**; oxo, **4**; or thio, **5**) or abolish (e.g. methylamino,**3**) binding.
3-Position	Exocyclic substituents decrease (e.g. oxide, **42**) or abolish (e.g. methyl, **43**) binding.
6-Position	Exocyclic oxo or thioxo group in the lactam tautomeric form is required for binding; substitution of 6-position oxo group of hypoxanthine (**1**) with a thio group (6-thiopurine, **11**) decreases binding slightly (1.7- and 14-fold‡, respectively); elimination (i.e. purine, **6**) or substitution of the 6-position oxo group decreases (e.g. amino, **7**; methylamino, **8**; benzylamino, **9**; or chloro, **12**) or abolishes (e.g. iodo, **13**; or methylk, **14**) binding.
7-Position	A pyridine-type nitrogen is preferred for binding; replacement with methine group (e.g. 7-deazaguanine, **31**) decreases binding slightly (2.2- and 9.3-fold‡, respectively). Substitution of an exocyclic methyl group (e.g. 7-methylguanine, **32**; or 7-methylxanthine, **33**) decreases binding by at least 136-fold.
8-Position	Endocyclic methine group is required for binding; replacement with nitrogen markedly decreases (e.g. 8-azahypoxanthine, **15**; or 8-azaguanine, **34**) or abolishes (8-azaxanthine, **36**) binding. Exocyclic substituents (e.g. oxo, **16**; thio, **17**; or bromo, **35**) markedly decrease (at least 120-fold) or abolish binding.
9-Position	Endocyclic pyrrole-type nitrogen or a methine carbon (e.g. 9-deazaguanine, **44**) is required for binding. Substitution of an exocyclic methyl group (e.g. 9-methylguanine, **45**) decreases binding (19- and 61-fold‡).

* Refers to the purine numbering system shown in Fig. 1.
† Bold type numbers refer to compound numbers in Table 3.
‡ Refers to XPRTase and GPRTase, respectively.

above, decreases the binding of purines to *T. gondii* XPRTase and GPRTase. Similarly, the lack of binding of 2-aminoimidazole (**78**) could be attributed to the existence of an amino group at the 8-position or the complete absence of the pyrimidine ring.

Activities of XPRTase and GPRTase in T. gondii

Competition studies between the various substrates (Table 2) suggested that more than one phosphoribosyltransferase may be involved in xanthine anabolism. This suggestion is strengthened further by the results shown in Table 3. Since the substrate concentrations used, 10 and 4 μM, were approximately 3-fold the K_m values for xanthine (3.0 ± 0.4 μM) and guanine (1.3 ± 0.4 μM), respectively, and the Michaelis–Menten equation for competitive inhibition is

$$v/V_{max} = [S]/\{K_m\,(1 + [I]/K_i) + [S]\}$$

substituting $[S] = 3\,K_m$ and solving the equation gives the following relationship:

$$v/V_{max} = (3/[I])/(1/K_i + 4/[I])$$

where the ratio v/V_{max} is a function of [I]. Therefore, using identical inhibitor concentrations for the same compound in both assays should result in identical K_i values if XPRTase and GPRTase were carried by a single enzyme activity. However, examination of Table 3 shows that 6-thiopurine (**11**), 1-methylguanine (**18**), isoguanine (**23**), 7-deazaguanine (**31**), and allopurinol (**47**), among others, exhibit markedly different K_i values between XPRTase and GPRTase. The majority of these compounds are guanine derivatives and yet they are better inhibitors of XPRTase, even though the xanthine concentration used was 2.5-fold greater than that of guanine. These findings together with the observed noncompetitive inhibition of hypoxanthine with XPRTase (Table 2) strongly indicate that XPRTase activity in *T. gondii* may be carried out by two different enzymes. However, definitive proof of this suggestion must wait until the isolation and characterization of purine phosphoribosyltransferases from *T. gondii* are accomplished.

Design of ligands to XPRTase and GPRTase from T. gondii

The structure–activity relationships for the binding of purine nucleobase analogues to *T. gondii* XPRTase and GPRTase are summarized in Table 4. On the basis of these findings, we propose the synthesis and testing of the following purine nucleobases as potential ligands for *T. gondii* XPRTase and GPRTase: 7-deazahypoxanthine, 7-deazaxanthine, 7-deaza-6-thiopurine, 7-deaza-2-amino-6-thiopurine and 7-deaza-2-oxo-6-thiopurine.

We are planning to test some of the more promising analogues that preferentially bind to *T. gondii* XPRTase (e.g. 2-aminopurine, **20**) as antitoxoplasmic agents. The rationale is that such analogues will selectively interfere with the purine salvage in the parasite but notthe host, since the host does not have XPRTase ([11–13], and unpublished results). Furthermore, the selective disruption of the purine salvage pathways in these parasites by analogues that are activated specifically by XPRTase would be enhanced further by the fact that toxoplasma, unlike their host, lack *de novo* purine biosynthesis, and are totally dependent on their salvage pathways for their vital purine requirements. Finally, the use of analogues that are activated by XPRTase as chemotherapeutic drugs would not be limited to the treatment of toxoplasmosis. Like *T. gondii*, several of the

pathogenic protozoa, including *Entamoeba histolytica* [27], *Eimeria tenella* [28], *Leishmania donovani* [29], *Plasmodium falciparum* [13, 30], *Tritrichomonas foetus* [31, 32], and *Trypanosoma cruzi* [33], lack purine *de novo* biosynthesis and have phosphoribosyltransferase activities that utilize xanthine as a substrate. Therefore, it is quite likely that other pathogenic protozoa will share the same ability of metabolizing xanthine, and if an effective anti-toxoplasmosis drug emerges from our ongoing screening, it is likely to be selectively effective against other pathogens of protozoal diseases.

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