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STRUCTURE–ACTIVITY RELATIONSHIP FOR THE BINDING OF NUCLEOSIDE LIGANDS TO ADENOSINE KINASE FROM *TOXOPLASMA GONDII*

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Abstract—One hundred and twenty-eight purine nucleoside analogs were evaluated as ligands of *Toxoplasma gondii* adenosine kinase (EC 2.7.1.20) by examining their ability to inhibit this enzyme *in vitro*. Inhibition was quantified by determining apparent K_i (appK_i) values for those compounds that inhibited this enzyme by greater than 10% at a concentration of 1 mM. Two compounds, N^6-(*p*-methoxybenzoyl)adenosine and 7-iodo-7-deazaadenosine (iodotubercidin), were found to bind to the enzyme (appK_i = 3.9 and 1.6 μM, respectively) better than adenosine. On the basis of these data, a structure–activity relationship for the binding of ligands to *T. gondii* adenosine kinase was formulated using adenosine as a reference compound. It was concluded that the following structural features of purine nucleoside analogs are required or strongly preferred for binding: (1) "pyridine-type" endocyclic nitrogens at the 1- and 3-positions; (2) an exocyclic hydrogen at the 2-position; (3) 6-position exocyclic substituents in the lactim tautomeric form; (4) a "pyridine-type" endocyclic nitrogen at the 7-position or hydrophobic exocyclic substituents attached to an endocyclic carbon at the 7-position; (5) an endocyclic methine or "pyridine-type" nitrogen at the 8-position; (6) an endocyclic nitrogen at the 9-position; (7) a pentose or "pentose-like" (e.g. hydroxylated cyclopentene) moiety attached to the 9-position nitrogen; (8) hydroxyl groups at the 2′- and 3′-positions in a ribose configuration; (9) a hydroxymethyl or methyl (i.e. 5′-deoxy) group at the 5′-position; (10) a β-D-nucleoside configuration; and (11) an *anti* conformation around the *N*-glycosidic bond. In addition, there appears to be a "pocket" in the catalytic site of *T. gondii* adenosine kinase, adjacent to the 6-position of adenosine, that can accommodate large (preferably unsaturated or aromatic) substituents (e.g. phenyl). These findings provide the basis for the rational design of additional ligands of *T. gondii* adenosine kinase.

Key words: Toxoplasma gondii; adenosine; kinase; structure–activity; ligands; inhibitors

Toxoplasma gondii is an obligate intracellular protozoa that infects humans and many species of warm-blooded animals [1]. *T. gondii* infections are usually asymptomatic in immunocompetent hosts; however, these parasites can cause severe and often fatal disease in immunocompromised individuals such as those suffering from AIDS§. In such patients, *T. gondii* is the most commonly recognized cause of opportunistic infection of the central nervous system, with clinically apparent infections reported to occur in 3–40% of these patients [2, 3]. The current standard therapy for *T. gondii* infections in AIDS patients is a combination of pyrimethamine and sulfadiazine [2, 4]. The initial response rate to this therapy is relatively high; however, side-effects such as bone marrow suppression and severe skin rashes force discontinuance of therapy in over half of all AIDS patients treated with this regimen [2, 4]. Thus, new chemotherapeutic targets and agents for the treatment of this disease need to be identified.

One potential chemotherapeutic target in *T. gondii* is purine nucleotide metabolism. In general, purine nucleotides can be synthesized by either *de novo* pathways, which use simple precursor molecules, or by the salvage pathways, which use preformed purine nucleobases or nucleosides from either endogenous or exogenous sources [5]. Unlike their hosts, *T. gondii* are incapable of *de novo* purine biosynthesis [6, 7] and must satisfy their requirement for vital purines by salvaging preformed purines from the host cell. Therefore, it may be possible to selectively deprive *T. gondii* of purine nucleotides by blocking or disrupting their purine salvage pathways.

Adenosine is incorporated into the nucleotide pools of *T. gondii* at a 10-fold higher rate than any other purine nucleobase or nucleoside [6]. In addition, adenosine is a likely source of purines for this parasite, given the high intracellular levels of ATP in their host cells [5]. The major route of adenosine metabolism in *T. gondii* is direct phosphorylation to AMP, from which all other purine nucleotides can be synthesized [6]. This reaction is catalyzed by the enzyme adenosine kinase (EC 2.7.1.20), which is approximately 10-fold more

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§ Abbreviations: AIDS, acquired immunodeficiency syndrome; appK_i, apparent K_i; EHNA, *erythro*-9-(2-*S*-hydroxy-3-*R*-nonyl)adenine; and ara-A, adenine arabinoside or 9-(β-D-arabinofuranosyl)adenine.

active than any other purine salvage enzyme in these parasites [6]. This contrasts sharply with mammalian cells where adenosine is deaminated to inosine, which is then cleaved to hypoxanthine by the sequential reactions of adenosine deaminase (EC 3.5.4.4) and purine nucleoside phosphorylase (EC 2.4.2.1), respectively [5]. Neither of these two enzymes has appreciable activity in *T. gondii* [6]. The high activity of adenosine kinase, along with the unique characteristics of adenosine metabolism in *T. gondii*, makes adenosine kinase an excellent target for chemotherapy.

Adenosine kinase in *T. gondii* could be exploited as a chemotherapeutic target in at least two ways. First, inhibition of this enzyme by nucleoside analogs would at least partially inhibit purine salvage in *T. gondii*. Second, analogs that are substrates for *T. gondii* adenosine kinase may be preferentially metabolized to toxic nucleotides in the parasite. One example of this type of analog is the compound ara-A, which has been shown to inhibit the growth of *T. gondii* in mammalian cells [8]. The "target" for this analog appears to be adenosine kinase, since ara-A resistant mutants have less than 0.1% of the adenosine kinase activity of the wild-type parasite [9]. It is thought that the resistance to ara-A is due to a lack of phosphorylation of this analog (by adenosine kinase) to an active form [9].

In the present study, one hundred and twenty-eight purine nucleoside analogs were evaluated as ligands of *T. gondii* adenosine kinase 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* adenosine kinase by greater than 10% at a concentration of 1 mM. On the basis of these data, a structure–activity relationship for the binding of purine nucleoside analogs to *T. gondii* adenosine kinase was formulated, in order to identify and/or design analogs that may bind specifically to the parasite enzyme.

MATERIALS AND METHODS

Chemicals and supplies

The sources of the compounds screened as inhibitors of adenosine kinase are indicated in Table 1 by the following abbreviations: ALD, Aldrich Chemical Co., Inc., Milwaukee, WI; AMC, American Cyanamid Co., Lederle Laboratories, Pearl River, NY; BWC, Burroughs Wellcome Co., Research Triangle Park, NC; CAL, Calbiochem, San Diego, CA; FLU, Fluka Chemical Co., Ronkonkoma, NY; ICN, ICN Biomedicals, Inc., Costa Mesa, CA; GG, Dr. Gilles Gosselin, Université de Montpellier, Montpellier, France; JM, Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL; LT, Dr. Leroy B. Townsend, University of Michigan, Ann Arbor, MI; NCI, Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD; RBI, Research Biochemicals International, Natick, MA; RK, Dr. Robert S. Klein, Montefiore Medical Center, Bronx, NY; RS, Dr. Raymond Schinazi, VA Medical Center, Emory University, Decatur, GA; SCH, Schweizerhall, Inc., Piscataway, NJ; SC, Dr. Shih Hsi Chu, Brown University, Providence, RI; SIG, Sigma Chemical Co., St. Louis, MO; and VM, Dr. Victor E. Marquez, National Cancer Institute, Bethesda, MD. [8-^{14}C]Adenosine (45 Ci/mol) 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. (+)-EHNA was a gift from Dr. Elie Abushanab, University of Rhode Island, Kingston, RI. 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 [10].

Preparation of cytosol extracts

Approximately 5×10^8 *T. gondii* were suspended in 1.2 mL of 50 mM HEPES-Cl (pH 8.0)/1 mM dithiothreitol and homogenized 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 and used as the enzyme source. Fresh cytosol extracts were prepared for each experiment.

Adenosine kinase assay

T. gondii adenosine kinase activity was measured by following the formation of [^{14}C]AMP from [^{14}C]-adenosine and ATP. Our initial studies (results not shown) to optimize the assay conditions indicated that the apparent K_m for adenosine was approximately 5 μM, the optimal concentration for $MgCl_2$ and ATP was 0.5 mM each, and the optimal pH was 8.0. It should be noted that ATP containing vanadium (~40 ppm) was used in all assays because the use of "vanadium-free" ATP resulted in low enzyme activity. We also found that the addition of 25 μM (+)-EHNA or 100 μM (±)-EHNA was required to inhibit any contaminating adenosine deaminase activity that may degrade the substrate adenosine to inosine. Therefore, the standard reaction mixture contained 50 mM HEPES-Cl (pH 8.0), 1 mM dithiothreitol, 5 μM [8-^{14}C]adenosine (45 Ci/mol), 0.5 mM ATP, 0.5 mM $MgCl_2$, 25 μM (+)-EHNA or 100 μM (±)-EHNA, 10–30 μL of cytosol extract (approximately 3–10 μg of protein), and either 0, 0.1, 0.25, 0.5 or 1.0 mM of the compound to be tested, in a final volume of 150 μL. To more accurately determine appK_i values, lower concentrations of the compound to be tested were used for very potent inhibitors or inhibitors that were poorly soluble, whereas higher concentrations were used for poor inhibitors. When compounds **9, 21, 22, 42, 47** and **48** (see Table 1) were tested, reaction mixtures (including those with no compound) contained 10% dimethyl sulfoxide (in addition to the standard

reaction mixture) to ensure the solubility of these compounds.

Reactions were started by the addition of [^{14}C]-adenosine, incubated at 37° for 10 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 AMP and adenosine. This mixture was then spotted on silica gel thin-layer chromatography plates that were developed with a mixture of chloroform: methanol: acetic acid (16:3:1). The average R_f values for AMP and adenosine were 0 and 0.37, respectively. The substrate and product spots (which accounted for all of the radioactivity on the plates) were identified by UV quenching and cut out, and the radioactivity was quantified by liquid scintillation counting in 20 mL of Scintilene using a Packard 460 scintillation counter. Under these conditions, velocity was linear with respect to time and amount of cytosol extract. Enzyme velocity was calculated by multiplying the fraction of AMP formed from adenosine times the amount of adenosine in the assay, and dividing by the incubation time.

Determination and significance of appK_i *values*

AppK_i values were used to determine the relative degree of binding of compounds to *T. gondii* adenosine kinase as compared with adenosine. AppK_i values were estimated from Dixon plots of the data [$1/v$ versus (I)] using a computer program that employs least-squares fitting according to 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 adenosine, appK_i values are related to K_i values by the following equation [12]: app$K_i = K_i(1 + (S)/K_m)$. In the present study, the concentration of adenosine (5 μM) was equal to its estimated K_m value. Thus, the appK_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, nor were the compounds evaluated as substrates for *T. gondii* adenosine kinase.

Protein determinations

Protein concentrations were determined by the method of Bradford [13] using the Bio-Rad Laboratories protein assay kit and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Evaluation of purine nucleoside analogs as ligands of T. gondii *adenosine kinase*

Three types of purine nucleoside analogs were screened in this study: (1) analogs with modifications to the purine moiety; (2) analogs with modifications to the pentose moiety; and (3) analogs with modifications to both the purine and pentose moieties. Primarily β-D-adenosine analogs were tested since the poor binding (see Table 1) of β-L-adenosine (**2**) and α-D-adenosine (**3**) indicated that *T. gondii* adenosine kinase is specific for β-anomers and D-isomers. These analogs were evaluated as ligands of *T. gondii* adenosine kinase 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* adenosine kinase by greater than 10% at a concentration of 1 mM. The mean and range of the appK_i values for these compounds, determined from at least two separate estimations, are shown in Table 1.

Binding of ligands to T. gondii *adenosine kinase*

The structure of adenosine and the numbering system for the purine and pentose moieties are shown in Fig. 1. Adenosine can theoretically exist in either an imino (lactam) or amino (lactim) tautomeric form; however, evidence indicates that the amino form predominates [14]. Furthermore, adenosine is fully ionized (i.e. lacks protons at positions 1, 3 and 7 of the purine ring) under the conditions employed in the present study [15]. The results shown in Table 1 will be discussed with respect to the effect of modifications to adenosine on binding to *T. gondii* adenosine kinase. To simplify the discussion, the purine ring numbering system (Fig. 1) will be used for all compounds, and compounds will be referred to by their "trivial" names and compound number (see Table 1).

1-*Position substitutions.* The endocyclic 1-position nitrogen of the purine ring is required for binding to *T. gondii* adenosine kinase, as replacement with a methine (CH) group abolished binding (e.g. 1-deazaadenosine, **5**; or 1-deazapurine riboside, **61**). The difference between an endocyclic ("pyridine-type") nitrogen and a methine group is that the nitrogen has an unshared pair of electrons that are not involved in the aromaticity of the heterocycle and are available for hydrogen bonding. Thus, the lack of binding of "1-deaza" analogs indicates that hydrogen bonding between the 1-position nitrogen

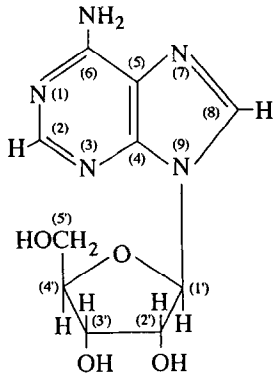

Fig. 1. Structure of the reference compound adenosine with numbering system for the purine and pentose moieties.

Table 1. AppK_i values for inhibition of *T. gondii* adenosine kinase

	Compound*	Source	AppK_i† (μM)
1	β-D-Adenosine	SIG	7.0 ± 3.3
2	β-L-Adenosine	RS	‡
3	α-D-Adenosine	SIG	8,400 ± 2,200
	Modifications in the purine moiety		
	1-Position substitutions		
4	Adenosine-N^1-oxide	SIG	1,230 ± 10
5	1-Deazaadenosine	BWC	‡
6	1-Methyladenosine	SIG	88 ± 28
	2-Position substitutions		
7	2-Chloroadenosine	SIG	870 ± 50
8	2-Fluoroadenosine	SC	3,000 ± 500
9	2-Phenylaminoadenosine	RBI	‡
	3-Position substitutions		
10	3-Deazaadenosine	BWC	‡
	6-Position substitutions		
11	Purine riboside (nebularine)	SIG	170 ± 20
12	6-Bromopurine riboside	NCI	28 ± 2
13	6-Chloropurine riboside	SIG	9.5 ± 0.3
14	6-Iodopurine riboside	NCI	18 ± 1
15	6-Oxopurine riboside (inosine)	SIG	‡
16	6-Methoxypurine riboside	SIG	370 ± 40
17	6-Thiopurine riboside (6-meracaptopurine riboside)	SIG	5,300 ± 400
18	6-Methylmercaptopurine riboside	SIG	22 ± 1
19	6-Ethylmercaptopurine riboside	NCI	330 ± 3
20	6-Benzylmercaptopurine riboside	NCI	140 ± 10
21	6-(*p*-Nitrobenzyl)mercaptopurine riboside	NCI	140 ± 10
22	6-(7-Nitrobenzofuroxan-4-yl)metcaptopurine riboside	NCI	11 ± 1
23	6-Selenopurine riboside	SIG	130 ± 20
24	6-(*p*-Nitrobenzyl)selenopurine riboside	SIG	84 ± 46
	N^6-Position substitutions		
25	N^6-Hydroxyadenosine	SIG	160 ± 30
26	N^6-Methyladenosine	SIG	23 ± 1
27	N^6-Dimethyladenosine	SIG	55 ± 6
28	N^6-Ethyladenosine	BWC	100 ± 3
29	N^6-(2-Hydroxyethyl)adenosine	SIG	320 ± 50
30	N^6-Allyladenosine	BWC	230 ± 20
31	N^6-Propynyladenosine	NCI	150 ± 5
32	N^6-(*n*-Butyl)adenosine	BWC	220 ± 90
33	N^6-(3-Chloro-2-butenyl)adenosine	NCI	220 ± 9
34	N^6-(4-Hydroxy-3-methyl-*trans*-2-butenyl)adenosine	SIG	280 ± 20
35	N^6-(*n*-Pentyl)adenosine	BWC	140 ± 10
36	N^6-(2-Isopentenyl)adenosine	SIG	51 ± 4
37	N^6-Cyclopentyladenosine	SIG	350 ± 10
38	N^6-Furfuryladenosine (kinetin riboside)	SIG	180 ± 10
39	N^6-(*n*-Hexyl)adenosine	BWC	76 ± 4
40	N^6-Cyclohexyladenosine	SIG	240 ± 70
41	N^6-(*n*-Decyl)adenosine	BWC	380 ± 130
42	N^6-(3,7-Dimethyl-2,6-octadienyl)adenosine	NCI	100 ± 5
43	N^6-Phenyladenosine	BWC	88 ± 12
44	N^6-Benzyladenosine	SIG	100 ± 30
45	N^6-(*p*-Aminobenzyl)adenosine	SIG	120 ± 10
46	N^6-Phenylethyladenosine	BWC	390 ± 240
47	(*R*)-N^6-(2-Phenylisopropyl)adenosine	RBI	750 ± 30
48	(*S*)-N^6-(2-Phenylisopropyl)adenosine	RBI	480 ± 70
49	N^6-Benzoyladenosine	SIG	23 ± 1
50	N^6-(*p*-Methoxybenzoyl)adenosine (N^6-anisoyladenosine)	SIG	3.9 ± 0.9
	7-Position substitutions		
51	7-Deazaadenosine (tubercidin)	SIG	340 ± 80
52	7-Chloro-7-deazaadenosine (chlorotubercidin)	NCI	10 ± 1
53	7-Iodo-7-deazaadenosine (iodotubercidin)	RBI	1.6 ± 0.1
54	7-Cyano-7-deazaadenosine (toyocamycin)	NCI	21 ± 2
55	7-Carboxamido-7-deazaadenosine (sangivamycin)	NCI	56 ± 4
56	7-Carboxamidoxime-7-deazaadenosine	NCI	200 ± 6

Table 1 continued.

	Compound*	Source	AppK_i† (μM)
	8-Position substitutions		
57	8-Azaadenosine	SIG	23 ± 6
58	8-Azidoadenosine	JM	520 ± 120
59	8-Bromoadenosine	SIG	660 ± 70
	9-Position substitutions		
60	9-Deazaadenosine	RK	3,400 ± 1,300
	1,6-Position substitutions		
61	1-Deazapurine riboside	BWC	‡
62	1,N^6-(Etheno)adenosine	SIG	4,300 ± 900
	2,6-Position substitutions		
63	2-Amino-6-chloropurine riboside	ALD	1,800 ± 100
64	2-Amino-6-hydroxypurine riboside (guanosine)	SIG	‡
65	(*R*)-2-Azido-N^6-(*p*-hydroxyphenylisopropyl)adenosine	ICN	59 ± 5
66	2-Chloro-N^6-cyclopentyladenosine	RBI	390 ± 90
67	2,6-Dihydroxypurine riboside (xanthosine)	SIG	‡
	6,7-Position substitutions		
68	6-Chloro-7-deazapurine riboside	NCI	72 ± 12
69	6-Hydrazino-7-deazapurine riboside	NCI	180 ± 10
70	N^6-Hydroxy-7-bromo-7-deazaadenosine	NCI	37 ± 9
71	N^6-Methyl-7-thiocarboxamido-7-deazaadenosine	NCI	150 ± 1
	7,8-Position substitutions		
72	8-Aza-7-deazaadenosine	NCI	1,700 ± 50
73	8-Bromo-7-carboxamidoxime-7-deazaadenosine	NCI	210 ± 30
74	8-Hydrazino-7-carboxamido-7-deazaadenosine	NCI	160 ± 9
	6,7,8-Position substitutions		
75	8-Aza-7-deazapurine riboside	BWC	5,100 ± 400
76	8-Aza-6-hydroxy-7-deazapurine (allopurinol) riboside	SIG	15,000 ± 1,000
77	8-Aza-6-mercapto-7-deazapurine riboside	BWC	‡
78	8-Aza-6-methylmercapto-7-deazapurine riboside	BWC	140 ± 30
79	8-Aza-6-ethylmercapto-7-deazapurine riboside	BWC	210 ± 100
	Other substitutions		
80	8-Aza-9-deazaadenosine (formycin A)	SIG	1,400 ± 200
81	8-Aza-9-deazainosine (formycin B)	SIG	3,100 ± 200
82	8-Aza-6-methylmercaptopurine riboside	BWC	130 ± 40
83	Benzimidazole riboside ("1,3-dideazapurine riboside")	BWC	‡
84	7-Carboxamido-7-deazaadenosine-N^1-oxide	NCI	780 ± 70
85	Coformycin	CAL	2,000 ± 900
	Modifications in the pentose moiety		
	2'-Position substitutions		
86	Adenine arabinoside (ara-A)	SCH	3,000 ± 700
87	2'-Deoxyadenosine	SIG	4,200 ± 1,000
88	2'-*O*-Methyladenosine	SIG	‡
89	2'-Tosyladenosine	SIG	‡
	3'-Position substitutions		
90	Adenine xyloside	GG	1,400 ± 100
91	3'-Deoxyadenosine (cordycepin)	SIG	1,100 ± 100
92	3'-*O*-Methyladenosine	SIG	‡
	5'-Position substitutions		
93	5'-Deoxyadenosine	SIG	40 ± 3
94	5'-Amino-5'-deoxyadenosine	SIG	220 ± 40
95	5'-Chloro-5'-deoxyadenosine	SIG	210 ± 10
96	5'-Iodo-5'-deoxyadenosine	SIG	230 ± 10
97	5'-Isobutyl-5'-deoxyadenosine	SIG	410 ± 90
98	5'-Methylthio-5'-deoxyadenosine	SIG	530 ± 230
99	5'-Carboxyladenosine (adenosine 5'-carboxylic acid)	SIG	1,100 ± 160
100	5'-*N*-(Methyl)carboxamidoadenosine	RBI	‡
101	5'-*N*-(Ethyl)carboxamidoadenosine	RBI	‡
102	5'-*N*-(Cyclopropyl)carboxamidoadenosine	SIG	1,100 ± 40
103	5'-Succinyladenosine (adenosine 5'-succinate)	SIG	920 ± 600
104	5'-Phosphoryladenosine (AMP)	SIG	1,000 ± 200
105	5'-Sulfonyladenosine (adenosine 5'-monosulfate)	SIG	210 ± 60
106	5'-(*p*-Fluorosulfonylbenzoyl)adenosine	FLU	340 ± 40

Table 1 continued.

	Compound*	Source	AppK_i† (μM)
	Multiple substitutions		
107	Adenine lyxoside	NCI	1,300 ± 70
108	2′,3′-Dideoxyadenosine	SIG	5,500 ± 2,900
109	2′,3′-*O*-*p*-Anisylidene adenosine	SIG	1,400 ± 200
110	2′,3′-Di-*O*-acetyladenosine	SIG	‡
111	3′,5′-Di-*O*-acetyladenosine	SIG	‡
112	4′-Fluoro-5′-*O*-sulfamoyladenosine (nucleocidin)	AMC	850 ± 220
	Other modifications		
113	Carbocyclic adenosine	JM	170 ± 30
114	Neplanocin A	NCI	37 ± 10
115	(±)-EHNA	SIG	‡
116	Adenine	SIG	6,500 ± 2,600
	Modifications in the purine and pentose moieties		
117	1-Methyl-2′-deoxyadenosine	SIG	8,000 ± 300
118	N^6-Methyl-2′-deoxyadenosine	SIG	‡
119	N^6-Benzoyl-2′-deoxyadenosine	FLU	590 ± 20
120	N^6-Dimethyl-3′-amino-3′-deoxyadenosine	SIG	540 ± 80
121	N^6-Dimethyl-3′-(α-amino-*p*-methoxyhydrocinnamamido)-3′-deoxyadenosine (puromycin)	SIG	1,300 ± 300
122	7-Carboxamido-7-deaza-3′-deoxyadenosine (3′-deoxysangivamycin)	LT	110 ± 20
123	2′-Fluoro-2′-deoxyguanosine	SIG	‡
124	2′-3′-*O*-Isopropylidene-6-mercaptopurine riboside	SIG	3,900 ± 500
125	5-Aminoimidazole-4-carboxamide riboside	SIG	2,400 ± 200
126	DDH-54	VM	‡
127	DDH-55	VM	‡
128	DDH-57	VM	940 ± 110

* Trivial names of compounds are listed with alternative names in parentheses. Chemical names of selected analogs are as follows: 1-deazaadenosine (**5**), 7-amino-3-(β-D-ribofuranosyl)imidazo[4,5-*b*]-pyridine; 3-deazaadenosine (**10**), 4-amino-1-(β-D-ribofuranosyl)imidazo[4,5-*c*]pyridine; 7-deazaadenosine (**51**), 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; 8-azaadenosine (**57**), 7-amino-3-(β-D-ribofuranosyl)-*v*-triazolo[4,5-*d*]pyrimidine; 9-deazaadenosine (**60**), 4-amino-7-(β-D-ribofuranosyl)-pyrrolo[3,2-*d*]pyrimidine; 8-aza-7-deazaadenosine (**72**), 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]-pyrimidine; 8-aza-9-deazaadenosine (**80**), 7-amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine; coformycin (**85**), 3-(β-D-ribofuranosyl)-6,7,8-trihydroimidazo[3,4-*d*](1,3)diazepin-8-(*R*)ol; adenine xyloside (**90**), 9-(β-D-xylofuranosyl)adenine; adenine lyxoside (**107**), 9-(β-D-lyxofuranosyl)adenine; carbocyclic adenosine (**113**), 9-(3-hydroxymethyl-4,5-dihydroxycyclopentan-1-yl)adenine; neplanocin A (**114**), 9-(3-hydroxymethyl-4,5-dihydroxy-2-cyclopenten-1-yl)adenine; DDH-54 (**126**), 4-(3-hydroxymethyl-4,5-dihydroxy-2-cyclopenten-1-yl)amino-5-nitro-6-aminopyrimidine; DDH-55 (**127**), 4-(3-hydroxymethyl-4,5-dihydroxy-2-cyclopenten-1-yl)amino-1,6-dihydro-5-nitro-6-oxopyrimidine; and DDH-57 (**128**), 2-amino-4-(3-hydroxymethyl-4,5-dihydroxy-2-cyclopenten-1-yl)amino-1,6-dihydro-5-nitro-6-oxopyrimidine.

† AppK_i values ± the range were obtained from at least two separate estimations of the appK_i.

‡ Less than 10% inhibition at a concentration of 1 mM.

and the catalytic site of *T. gondii* adenosine kinase plays a significant role in the binding of purine nucleoside ligands.

Exocyclic substitutions at the 1-position decreased binding. For example, substitution of an exocyclic methyl group at the 1-position of adenosine (**1**) (1-methyladenosine, **6**) or 2′-deoxyadenosine (**87**) (1-methyl-2′-deoxyadenosine, **117**) diminished binding 13- and 2-fold, respectively, while substitution of an exocyclic oxide at the 1-position of adenosine (**1**) (adenosine-N^1-oxide, **4**) or 7-carboxamido-7-deazaadenosine (**55**) (7-carboxamido-7-deaza-adenosine-N^1-oxide, **84**) decreased binding 180- and 14-fold, respectively. It is important to note that exocyclic substituents at the 1-position decreased binding, whereas substitution of an endocyclic methine (CH) group at the 1-position (e.g. 1-deazaadenosine, **5**) abolished binding. These results indicate that the loss of hydrogen bonding with the 1-position nitrogen (as is the case for 1-deazaadenosine, **5**) has a much greater effect on binding than steric hindrance by exocyclic substituents (e.g. 1-methyladenosine, **6**). The very poor binding of adenosine-N^1-oxide (**4**), on the other hand, suggests that other factors may be involved. One possibility is charge repulsion due to the negative and positive charges localized on the exocyclic oxygen and endocyclic nitrogen, respectively.

2-Position substitutions. An exocyclic hydrogen at the 2-position is required for binding to *T. gondii* adenosine kinase. Substitution of the 2-position hydrogen with halogens, such as a chloro (2-chloroadenosine, **7**) or fluoro (2-fluoroadenosine, **8**) group, markedly decreased binding (120- and 430-fold, respectively). Fluorine is very similar to hydrogen with respect to its size [16] and hydrophobicity [17]; therefore, the decreased binding of 2-fluoroadenosine (**8**) probably is due to the electron-withdrawing effects of this group. This is supported by the fact that a fluoro group, which is more electron-withdrawing than a chloro group [18],

decreased binding to a greater extent. However, it should be noted that addition of a halogen to the 2-position did not always affect binding. For example, addition of a chloro group to the 2-position of N^6-cyclopentyladenosine (**37**) (2-chloro-N^6-cyclopentyladenosine, **66**) decreased binding only slightly (1.1-fold).

Substitution of the 2-position hydrogen with an amino group also diminished binding, as indicated by the decreased binding (190-fold) of 2-amino-6-chloropurine riboside (**63**) as compared with 6-chloropurine riboside (**13**), while substitution of a phenylamino group to adenosine (2-phenylaminoadenosine, **9**) abolished binding. The decreased binding of 2-phenylaminoadenosine can certainly be ascribed to steric hindrance by the substituent group. However, the basis for the decreased binding caused by an amino group at the 2-position is less clear given the fact that 2-azido-N^6-(*p*-hydroxyphenylisopropyl)adenosine (**65**) binds slightly (8.4-fold) less than adenosine. An azido group is certainly larger than an amino group and given the data for 6-position substitutions (see below), it is unlikely that an N^6-(*p*-hydroxyphenylisopropyl) group (not tested) would increase binding more than a 6-position chloro group (**13**). It should be noted, however, that purine analogs that have an azido group at the 2-position can form tetrazolo tautomers with the 1- or 3-position endocyclic nitrogens [19]. Although this would preclude hydrogen bonding with *N*1 or *N*3, it is possible that hydrogen bonding could occur between the nitrogens of the tetrazole group and the catalytic site of the enzyme. Regardless of the mechanism, the relatively good binding of 2-azido - N^6 - (*p* - hydroxyphenylisopropyl)adenosine (**65**) suggests that an azido group at the 2-position may enhance binding to the enzyme.

3-*Position substitutions.* The endocyclic 3-position nitrogen of adenosine is required for binding, as replacement with a methine group (3-deazaadenosine, **10**) abolished binding to *T. gondii* adenosine kinase. This suggests that hydrogen bonding between the unshared electrons of the 3-position nitrogen and the catalytic site of the enzyme plays an important role in the binding of purine nucleoside ligands, as suggested for the 1-position nitrogen (see above).

6-*Position substitutions.* An exocyclic amino group at the 6-position of the purine moiety (adenosine, **1**) is preferred for binding to *T. gondii* adenosine kinase. Elimination of the amino group of adenosine (purine riboside, **11**) decreased binding by about 25-fold. On the other hand, replacement of the amino group with halogens (i.e. bromo, **12**; chloro, **13**; or iodo, **14**) diminished binding only slightly (4-, 1.4- and 2.6-fold, respectively). No pattern was observed with respect to the size [16], electronegativity [18] or hydrophobicity [17] of these groups and their effect on binding. However, these compounds (**12, 13, 14**) exist as lactim tautomers (as is the case for adenosine), suggesting that the tautomeric form of these compounds plays a more important role in their binding to the enzyme than the nature of the 6-position substituents.

The significance of the tautomeric form of ligands of *T. gondii* adenosine kinase is supported further by the fact that substitution of the 6-position amino group of adenosine with an oxo group (inosine, **15**) abolished binding, while replacement with a thio group (6-thiopurine riboside, **17**) or seleno group (6-selenopurine riboside, **23**) decreased binding by about 750- and 19-fold, respectively. In contrast to adenosine, these compounds exist predominately as lactam (keto) tautomers, rather than lactim (enol) tautomers [20]. The percentage of the lactim form of 6-thiopurine riboside (**17**) is greater than that of inosine (**15**) (i.e. 7 vs 1%) [20], and it is possible that the percentage of the lactim form of 6-selenopurine riboside (**23**) is even greater than that of 6-thiopurine riboside (**17**). Thus, the binding of these compounds appears to be directly related to the percentage of the lactim tautomer, i.e. seleno (**23**) > mercapto (**17**) > hydroxy (**15**). Furthermore, substitutions to the 6-position of these compounds (i.e. **15, 17** and **23**) that increase the level of the lactim tautomer also increase binding. For example, addition of a methyl group to the 6-position oxo group of 6-oxopurine riboside (**15**) (6-methoxypurine riboside, **16**) improved binding from less than 10% inhibition (at 1 mM) to an appK_i of 370 μM. Similarly, addition of a methyl (**18**), ethyl (**19**), benzyl (**20**), *p*-nitrobenzyl (**21**) or nitrobenzofuroxan-4-yl (**22**) group to the 6-position thio group of 6-thiopurine riboside (**17**) also increased binding (240-, 16-, 38-, 38- and 480-fold, respectively). The better binding of the lactim forms of these compounds is probably due to the presence of a "pyridine-type" 1-position nitrogen, which appears to be involved in hydrogen bonding to the catalytic site of *T. gondii* adenosine kinase (see above).

N^6-*Position substitutions.* With one exception (N^6-(*p*-methoxybenzoyl)adenosine, **50**), all N^6-substitutions decreased binding to *T. gondii* adenosine kinase, relative to adenosine. However, some general trends were seen for these compounds with respect to their 6-position substituents and binding. For example, it was found that hydrophobic substituents (e.g. N^6-ethyladenosine, **28**) were preferred over hydrophilic substituents (e.g. N^6-(2-hydroxyethyl)adenosine, **29**). However, no definite correlation between the chain length of hydrophobic substituents and binding was observed, as the appK_i values for N^6-alkyl substituents were in the order: methyl (**26**) < *n*-hexyl (**39**) < ethyl (**28**) < *n*-pentyl (**35**) < *n*-butyl (**32**) < *n*-decyl (**41**). On the other hand, some consistent trends with respect to N^6-substituents were seen. For example, straight chain aliphatic substituents (e.g. N^6-(*n*-pentyl)adenosine, **35**) were preferred over cyclic aliphatic substituents (e.g. N^6-cyclopentyladenosine, **37**), unsaturated substituents (e.g. N^6-(2-isopentenyl)adenosine, **36**) were preferred over saturated substituents (e.g. N^6-(*n*-pentyl)adenosine, **35**), and aromatic substituents (e.g. N^6-phenyladenosine, **43**) were preferred over non-aromatic substituents (e.g. N^6-cyclohexyladenosine, **40**).

These results indicate that there is a "pocket" in the catalytic site of *T. gondii* adenosine kinase adjacent to the 6-position of adenosine, which is large enough to accommodate large aliphatic (e.g. *n*-decyl) or aromatic (e.g. phenyl) groups. This is similar to our previous findings [21] for *T. gondii*

uridine phosphorylase (EC 2.4.2.3), which indicated that there is a pocket in the catalytic site of this enzyme that is adjacent to the 5- and 6-positions of uracil. In the case of uridine phosphorylase, substitution of large hydrophobic groups (e.g. benzyl) at these positions (of uracil) greatly enhanced binding, indicating that hydrophobic interactions were involved. In contrast, substitution of hydrophobic groups at the 6-position of adenosine generally decreased binding to *T. gondii* adenosine kinase (relative to adenosine), indicating that there are no "strong" hydrophobic interactions involved.

When a variety N^6-phenyl-substituted adenosine analogs were tested, the linkage between the phenyl group and the 6-position nitrogen of adenosine was found to affect binding. Introduction of a methylene (N^6-benzyladenosine, **44**), ethylene (N^6-phenylethyladenosine, **46**), or isopropylene linkage (*R*-N^6-(2-phenylisopropyl)adenosine, **47**; or *S*-N^6-(2-phenylisopropyl)adenosine, **48**) decreased binding (1.1-, 4.4-, 8.5- and 5.5-fold, respectively) as compared with no linkage (N^6-phenyladenosine, **43**). Thus, binding decreased as the length and hydrophobicity of the linkage increased. It is of interest to note that the binding of the (*S*) configuration (**48**) of N^6-(2-phenylisopropyl)adenosine was 1.6-fold better than the (*R*) configuration (**47**), suggesting that the geometry of N^6-substituents plays a role in binding to the catalytic site of the enzyme. In contrast to the alkyl (i.e. methylene, ethylene, propylene) linkages, introduction of a carbonyl linkage (N^6-benzoyladenosine, **49**) increased binding (3.8-fold), rather than decreased binding. Since the difference between these linkages is the presence of an oxygen in the carbonyl linkage, it would appear that hydrophilic linkages are preferred over hydrophobic linkages. One possible explanation for this preference is that hydrogen bonding occurs between the carbonyl group and the catalytic site of the enzyme.

7-*Position substitutions.* The endocyclic 7-position nitrogen of adenosine is strongly preferred for binding to *T. gondii* adenosine kinase, as replacement with a methine group (7-deazaadenosine or tubercidin, **51**) decreased binding by about 49-fold. A decrease in binding (8- and 74-fold, respectively) was also observed for 6-chloro-7-deazapurine riboside (**68**) and 8-aza-7-deazaadenosine (**72**) relative to 6-chloropurine riboside (**13**) and 8-azaadenosine (**57**), respectively. As suggested for the 1- and 3-position endocyclic nitrogens (see above), the decreased binding of "7-deaza" compounds may be due to the lack of a "pyridine-type" nitrogen at the 7-position, which could be involved in hydrogen bonding to the catalytic site of the enzyme. However, in contrast to the 1- and 3-position nitrogens, substitution of the 7-position nitrogen with a methine group decreased binding, rather than abolished binding. In addition, several exocyclic substituents at the 7-position of 7-deazaadenosine (**51**) improved binding (see below), suggesting that hydrogen bonding to the 7-position nitrogen is not as critical for binding to the catalytic site of the enzyme as the 1- and 3-position nitrogens.

Exocyclic substitutions at the 7-position of 7-deazaadenosine (**51**) generally increased binding. For example, substitution of a cyano (toyocamycin, **54**), carboxamido (sangivamycin, **55**) or carboxamidoxime (**56**) group increased binding 16-, 6- and 1.7-fold, respectively, relative to 7-deazaadenosine (**51**). The carboxamido and carboxamidoxime substitutions were also observed to improve binding for other compounds as well. For example, 7-carboxamido-7-deazaadenosine-N^1-oxide (**84**) and 8-bromo-7-carboxamidoxime-7-deazaadenosine (**73**) were better ligands (1.6- and 3.1-fold) than adenosine-N^1-oxide (**4**) or 8-bromoadenosine (**59**), respectively. Substitution of exocyclic halogens at the 7-position of 7-deazaadenosine (**51**) such as chloro (**52**) or iodo (**53**) groups, resulted in an even greater increase in binding (34- and 210-fold, respectively). Although the compound 7-bromo-7-deazaadenosine was not available for testing, N^6-hydroxy-7-bromo-7-deazaadenosine (**70**) was found to bind better (4.3-fold) than N^6-hydroxyadenosine (**25**). If a similar increase in binding occurs for 7-bromo-7-deazaadenosine as compared with adenosine, the increase in binding due to these types of substitutions (i.e. "7-halo-7-deazaadenosine") would fall in the order iodo > bromo > chloro. This sequence is in the order of the hydrophobicities of these groups [17], indicating that binding increases as the hydrophobicity of the halogen substituent increases. Indeed, the binding of all of the exocyclic-substituted 7-deazaadenosine analogs was in the same order as the hydrophobicities [17] of the substituent groups (i.e. iodo > chloro > cyano > carboxamido > carboxamidoxime). These results suggest that there may be hydrophobic residues in the catalytic site of the enzyme near the 7-position of adenosine.

8-*Position substitutions.* The endocyclic 8-position carbon of adenosine is preferred but not required for binding, as replacement with a nitrogen (8-azaadenosine, **57**) decreased binding by only 3-fold. In addition, substitution of the 8-position carbon of 6-methylmercaptopurine riboside (**18**) (8-aza-6-methylmercaptopurine riboside, **82**) or 7-deazaadenosine (**51**) (8-aza-7-deazaadenosine, **72**), decreased binding by 6- and 5-fold, respectively. The similar binding of 8-azaadenosine (**57**) and adenosine (**1**) is not surprising since the endocyclic 1-, 3- and 7-position nitrogens, which appear to be critical for binding (see above), are all available for hydrogen bonding in these two compounds [22]. The slight decrease in binding observed for "8-aza" substitutions may be due to a change in the conformation of the nucleosides around the *N*-glycosidic bond. Compounds with "8-aza" substitutions (e.g. 8-azaadenosine, **57**; or formycin A, **80**) have been shown to exist in an intermediate conformation between *anti* and *syn* known as "high-*anti*" [23], whereas most purine nucleosides exist in the *anti* conformation [24].

Substitution of an exocyclic azido (8-azidoadenosine, **58**) or bromo (8-bromoadenosine, **59**) group to the 8-position of adenosine decreased binding markedly (75- and 94-fold, respectively). However, substitution of a bromo group to the 8-position of 7-carboxamidoxime-7-deazaadenosine (**56**) (8-bromo-7-carboxamidoxime-7-deazaadenosine, **73**), or a hydrazino group to the 8-position of

7-carboxamido-7-deazaadenosine (**55**) (8-hydrazino-7-carboxamido-7-deazaadenosine, **74**), decreased binding by only 1.1- and 3-fold, respectively. It has been shown that purine nucleosides with bulky substituents at the 8-position (e.g. 8-bromoadenosine, **59**) exist in the *syn* conformation rather than *anti* [24, 25]. Thus, the poor binding of 8-azidoadenosine (**58**) and 8-bromoadenosine (**59**) indicates that *T. gondii* adenosine kinase preferentially binds ligands in the *anti* conformation, and is consistent with the small decrease in binding observed for compounds in the "high-*anti*" conformation (see above). The conformation of 8-bromo-7-carboxamidoxime-7-deazaadenosine (**73**) and 8-hydrazino-7-carboxamido-7-deazaadenosine (**74**) is unknown. However, the presence of both endocyclic and exocyclic substitutions at the 7-position of these compounds may prevent the shift from the *anti* to the *syn* conformation, which would explain the slight decrease in binding observed for these analogs.

9-position substitutions. The endocyclic 9-position nitrogen of adenosine is required for binding as replacement with a carbon (9-deazaadenosine, **60**) markedly decreased binding (490-fold) to *T. gondii* adenosine kinase. Similarly, substitution of the 9-position nitrogen of 8-azaadenosine (**57**) (8-aza-9-deazaadenosine or formycin A, **80**) also decreased binding (61-fold). It has been shown that 8-aza-9-deazaadenosine (**80**) exists predominately (94%) as the *N*(H)7 tautomer [26, 27]. Thus, the poor binding of 8-aza-9-deazaadenosine (**80**) and 9-deazaadenosine (**60**) is probably due to the lack of a "pyridine-type" nitrogen at the 7-position, which appears to be involved in hydrogen bonding to the catalytic site of the enzyme (see above). The slightly better binding (2.4-fold) of 8-aza-9-deazaadenosine (**80**) versus 9-deazaadenosine (**60**) is probably due to the presence of a small amount (6%) of the *N*(H)8 tautomer [27] of 8-aza-9-deazaadenosine (**80**), whereas 9-deazaadenosine (**60**) probably exists exclusively as the *N*(H)7 tautomer.

Other substitutions to the purine moiety. With the exception of the 7- and 8-positions, it appears that an intact purine moiety (i.e. all of the endocyclic nitrogens and carbons in the correct position) is required for binding to *T. gondii* adenosine kinase. This is supported by the poor binding of compounds such as: benzimidazole riboside (**83**), which lacks the 1- and 3-position nitrogens; coformycin (**85**), which contains an extra carbon in the pyrimidine ring portion of the purine moiety; and 5-aminoimidazole-4-carboxamide riboside (**125**), which lacks an intact pyrimidine portion of the purine moiety. Similarly, the compounds DDH-54 (**126**), DDH-55 (**127**) and DDH-57 (**128**), which are analogs of neplanocin A (**114**) that lack an intact imidazole portion of the purine moiety, are also poor ligands of *T. gondii* adenosine kinase.

2′-Position substitutions. The 2′-hydroxyl group of adenosine appears to play an important role in binding to the catalytic site of *T. gondii* adenosine kinase, probably through hydrogen bonding. Elimination of the 2′-hydroxyl group (2′-deoxyadenosine, **87**) decreased binding 605-fold, while substitution of the 2′-hydroxyl group with a methoxy (2′-*O*-methyladenosine, **88**) or tosyl (2′-tosyladenosine, **89**) group abolished binding. This decrease in binding was also seen with other adenosine analogs. For example, 1-methyl-2′-deoxyadenosine (**117**), N^6-methyl-2′-deoxyadenosine (**118**), and N^6-benzoyl-2′-deoxyadenosine (**119**), all have higher appK_i values than their riboside counterparts (i.e. 1-methyladenosine, **6**; N^6-methyladenosine, **26**; and N^6-benzoyladenosine, **49**). In addition, changing the configuration of the 2′-hydroxyl group from the *ribo* to the *ara* configuration (ara-A, **86**) decreased binding 430-fold, indicating that *T. gondii* adenosine kinase prefers the 2′-hydroxyl group to be in the *ribo* configuration.

3′-Position substitutions. As with the 2′-hydroxyl group, *T. gondii* adenosine kinase prefers the 3′-hydroxyl group of adenosine to be in the *ribo* configuration, as a change to the *xylo* configuration (**90**) decreased binding markedly (200-fold). Elimination of the 3′-hydroxyl of adenosine (3′-deoxyadenosine, **91**) also decreased binding (150-fold), while substitution with a methoxy group (3′-*O*-methyladenosine, **92**) abolished binding. On the other hand, elimination of the 3′-hydroxyl of 7-carboxamido-7-deazaadenosine (**55**) (7-carboxamido-7-deaza-3′-deoxyadenosine, **122**) decreased binding by only 2-fold. Similarly, substitution of the 3′-hydroxyl of N^6-dimethyladenosine (**27**) with an amino group (N^6-dimethyl-3′-amino-3′-deoxyadenosine, **120**), decreased binding about 10-fold. Thus, it appears that the presence of the 3′-hydroxyl enhances binding, but may not be required for binding to *T. gondii* adenosine kinase.

5′-Position substitutions. Elimination of the 5′-hydroxyl group of adenosine (5′-deoxyadenosine, **93**) decreased binding by only 5.7-fold, suggesting that this group is not critical for binding to *T. gondii* adenosine kinase. Substitution of the 5′-hydroxyl group with an amino (**94**) group or halogens (chloro, **95**; iodo **96**), decreased binding approximately 30-fold, while substitution with hydrophobic (isobutyl, **97**; methylthio, **98**) groups reduced binding even more (59- and 76-fold, respectively). Similarly, groups at the 5′-position that are negatively charged (carboxyl, **99**; succinyl, **103**; phosphoryl, **104**) and/or relatively large (e.g. **100**, **101** and **102**) generally decreased or abolished binding. These results are consistent with a reaction mechanism in which both ATP and adenosine bind to *T. gondii* adenosine kinase. Thus, adenosine analogs with 5′-position groups that are large, hydrophobic, or negatively charged would bind poorly to the adenosine binding site due to steric hindrance (overlap) or charge repulsion by the phosphates on ATP. Two exceptions to this generalization are the compounds 5′-sulfonyladenosine (**105**) and 5′-(*p*-fluorosulfonylbenzoyl)adenosine (**106**), which bind better than compounds such as 5′-phosphoryladenosine (AMP, **104**) or 5′-succinyladenosine (**103**). One possible explanation for these exceptions is that these compounds bind to the ATP binding site on the enzyme, rather than the adenosine binding site.

Other substitutions to the pentose moiety. A pentose or "pentose-like" moiety in a *ribo* configuration is required for binding to *T. gondii* adenosine kinase. For example, compounds that lack a pentose moiety

(e.g. adenine, **116**; or EHNA, **115**) are very poor ligands. On the other hand, carbocyclic adenosine (**113**) and neplanocin A (**114**), which have hydroxylated cyclopentane and cyclopentene moieties, respectively, bind to *T. gondii* adenosine kinase. The importance of the 2′- and 3′-hydroxyl groups being present in a *ribo* configuration is supported by the poor binding of 2′,3′-dideoxyadenosine (**108**), which lacks both hydroxyl groups, and adenine lyxoside (**107**), which has the hydroxyl groups in a *lyxo* configuration.

Design of ligands of T. gondii *adenosine kinase*

The structure–activity relationship for the binding of purine nucleoside analogs to *T. gondii* adenosine kinase is summarized in Table 2. On the basis of these findings, several compounds (not currently

Table 2. Structure–activity relationship for the binding of nucleoside ligands to *T. gondii* adenosine kinase

Position*	Substituent effect
1-Position	Endocyclic "pyridine-type" nitrogen required; replacement with endocyclic methine group (1-deazaadenosine, **5**) abolishes binding.
2-Position	Exocyclic hydrogen required; substitution (e.g. chloro, **7**; fluoro, **8**; or amino, **63**) markedly decreases (120- to 430-fold) or abolishes (e.g. phenylamino, **9**) binding.
3-Position	Endocyclic "pyridine-type" nitrogen required; replacement with endocyclic methine group (3-deazaadenosine, **10**) abolishes binding.
6-Position	Exocyclic amino group preferred; elimination of group (purine riboside, **11**) decreases binding (25-fold).
	Exocyclic substituents in lactim tautomeric form strongly preferred; substitution of amino group with groups in lactim tautomeric form (e.g. bromo, **12**; chloro, **13**; or iodo, **14**) diminishes binding slightly (1.4- to 4-fold); substitution of amino group with groups in lactam tautomeric form (e.g. oxo **15**; thio, **17**; or seleno, **23**) decreases (19- to 750-fold) or abolishes binding.
N^6-Position	Hydrophobic substituents preferred over hydrophilic substituents, e.g. ethyl (**28**) vs 2-hydroxyethyl (**29**); straight chain aliphatic substituents preferred over cyclic aliphatic substituents, e.g. *n*-pentyl (**35**) vs cyclopentyl (**37**); unsaturated substituents preferred over saturated substituents, e.g. 2-isopentenyl (**36**) vs *n*-pentyl (**35**); aromatic substituents preferred over non-aromatic substituents, e.g. phenyl (**43**) vs cyclohexyl (**40**).
	Hydrophilic linkages (e.g. carbonyl, **49**) between aromatic groups and N^6 preferred over hydrophobic linkages (e.g. methylene, **44**).
7-Position	Endocyclic "pyridine-type" nitrogen strongly preferred; replacement with endocyclic methine group (7-deazaadenosine, **51**) decreases binding (49-fold).
	Hydrophobic exocyclic substituents (e.g. chloro, **52**; or iodo, **53**) attached to endocyclic carbon preferred over hydrophilic substituents (e.g. cyano, **54**; carboxamido, **55**; or carboxamidoxime, **56**).
8-Position	Endocyclic methine group preferred but not required; replacement with endocyclic nitrogen (8-azaadenosine, **57**) decreases binding slightly (3-fold).
9-Position	Endocyclic nitrogen required; replacement with endocyclic carbon (9-deazaadenosine, **60**) decreases binding markedly (490-fold).
2′-Position	Exocyclic 2′-hydroxyl group in *ribo* configuration required for binding; elimination (2′-deoxyadenosine, **87**) or substitution of group (e.g. methoxy, **88**) markedly decreases (600-fold) or abolishes binding; change in configuration of group to *ara* configuration (**86**) markedly decreases binding (430-fold).
3′-Position	Exocyclic 3′-hydroxyl group in *ribo* configuration strongly preferred for binding; elimination (3′-deoxyadenosine, **91**) or substitution of group (e.g. methoxy, **92**) markedly decreases (150-fold) or abolishes binding; change in configuration of group to *xylo* configuration (**90**) markedly decreases binding (200-fold).
5′-Position	Hydroxymethyl or methyl group strongly preferred for binding; elimination of hydroxyl group (5′-deoxyadenosine, **93**) decreases binding slightly (5.7-fold); substitution of hydroxyl group (with variety of groups, i.e. **94–106**) markedly decreases (at least 30-fold) or abolishes binding.
Other	A pentose or "pentose-like" moiety in a *ribo* configuration required for binding; elimination (i.e. adenine, **116**) of pentose moiety or a change to *lyxo* configuration (adenine lyxoside, **107**) markedly decreases binding (930- and 190-fold, respectively); replacement of pentose moiety with hydroxylated cyclopentene moiety (neplanocin A, **114**) decreases binding slightly (5.3-fold).
	β-D-Nucleoside configuration required; change in configuration to α-D-nucleoside (α-D-adenosine, **3**) markedly decreases binding (1200-fold); change in configuration to β-L-nucleoside (β-L-adenosine, **2**) abolishes binding.
	Anti conformation around *N*-glycosidic bond preferred; change to *syn* conformation (e.g. 8-bromoadenosine, **59**) decreases binding (94-fold).

* Refers to the numbering system for adenosine shown in Fig. 1.

available) can be proposed as potential ligands of *T. gondii* adenosine kinase. In particular, it would be of interest to test N^6-benzoyladenosine analogs with a methoxy group at either the *meta*- or *ortho*-position to see if the position of this group has any effect on binding. Similarly, N^6-benzoyladenosine analogs with larger (e.g. ethoxy) groups at the various positions (*meta, ortho* or *para*) should be tested to see if the size of the substitution increases or decreases binding. In addition, compounds with hydrophobic groups linked to N^6 through hydrophilic linkages other than a carbonyl linkage should be screened. At the 7-position, a series of 7-deazaadenosine (tubercidin) analogs that have exocyclic hydrophobic groups at the 7-position (e.g. 7-methyl-7-deazaadenosine) should be tested, in order to determine the optimal size and hydrophobicity of the 7-position substituent. With respect to the pentose moiety, the relatively good binding of neplanocin A (**114**) suggests that additional analogs with a hydroxylated cyclopentene attached to the purine ring should be tested. This substitution may improve the binding specificity of these compounds relative to other *T. gondii* (e.g. adenosine deaminase) or host (e.g. mammalian adenosine kinase) enzymes. Finally, 5′-deoxyadenosine analogs should be considered in the design of strict inhibitors of *T. gondii* adenosine kinase (i.e. no substrate activity), whereas substrate analogs should retain the 5′-hydroxyl group.

Summary

The present study has identified two compounds, N^6-(*p*-methoxybenzoyl)adenosine (**50**) and 7-iodo-7-deazaadenosine (iodotubercidin, **53**), that bind to *T. gondii* adenosine kinase (appK_i = 3.9 and 1.6 μM, respectively) better than adenosine. In addition, a structure–activity relationship for the binding of purine nucleoside analogs to *T. gondii* adenosine kinase has been established, which will provide the basis for the rational design of additional nucleoside ligands of this enzyme. These ligands represent potential chemotherapeutic agents that could either inhibit *T. gondii* adenosine kinase, or be metabolized by this enzyme to toxic nucleotides as in the case of ara-A [9]. Therefore, future studies will include the screening of selected compounds (particularly the N^6- and 7-substituted analogs) against *T. gondii*-infected mammalian cells, in order to ascertain their usefulness as antitoxoplasmal agents.

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