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6-Benzylthioinosine analogues as subversive substrate of *Toxoplasma gondii* adenosine kinase: Activities and selective toxicities

Reem H. Rais [a], Omar N. Al Safarjalani [a], Vikas Yadav [b], Vincenzo Guarcello [a], Marion Kirk [a], Chung K. Chu [b], Fardos N.M. Naguib [a], Mahmoud H. el Kouni [a,*]

[a] *Department of Pharmacology and Toxicology, Center for AIDS Research, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA*
[b] *Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA 30605, USA*

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Abstract

Toxoplasma gondii adenosine kinase (EC.2.7.1.20) is the major route of adenosine metabolism in this parasite. The enzyme is significantly more active than any other enzyme of the purine salvage in *T. gondii* and has been established as a potential chemotherapeutic target for the treatment of toxoplasmosis. Certain 6-substituted purine nucleosides act as subversive substrates of *T. gondii*, but not the human, adenosine kinase. Therefore, these compounds are preferentially metabolized to their respective nucleotides and become selectively toxic against the parasites but not their host. Herein, we report the testing of newly synthesized 6-benzylthioinosine analogues with various substituents on the phenyl ring of their benzyl group as subversive substrates of *T. gondii* adenosine kinases. The binding affinity of these compounds to *T. gondii* adenosine kinase and their efficacy as antitoxoplasmic agents varied depending on the nature and position of the various substituents on the phenyl ring of their benzyl group. *p-Cyano-6-benzylthioinosine* and 2,4-dichloro-6-benzylthioinosine were the best ligands. In general, analogues with substitution at the *para* position of the phenyl ring were better ligands than those with the same substitutions at the *meta* or *ortho* position. The better binding of the *para*-substituted analogues is attributed to the combined effect of hydrophobic as well as van der Waals interactions. The 6-benzylthioinosine analogues were devoid of host-toxicity but all showed selective anti-toxoplasmic effect in cell culture and animal models. These results further confirm that toxoplasma adenosine kinase is an excellent target for chemotherapy and that 6-substituted purine nucleosides are potential selective antitoxoplasmic agents

Keywords: Toxoplasma; Adenosine kinase; Subversive substrates; 6-Benzylthioinosine analogues; Chemotherapy

1. Introduction

Toxoplasma gondii is an obligate intracellular parasitic protozoan that infects humans and many species of warm-blooded animals [1]. Approximately a billion people worldwide, including 60% of the population in the United States, are seropositive to *T. gondii*. Infection with *T. gondii* is asymptomatic (90% of cases) in the general population. However, toxoplasmosis represents a major health problem for immunocompromised individuals, such as AIDS patients, organ transplant recipient patients, and the unborn children of infected mothers [1–4]. In such cases, toxoplasmic encephalitis is recognized as the most common cause of intracerebral mass lesions in AIDS patients and possibly the most commonly recognized opportunistic infection of the central nervous system [2,3]. Congenital toxoplasmosis is as high as 1/1000 live births [3]. Effects range in severity from asymptomatic to stillbirth, with the most common ailments being retinochoroiditis, cerebral calcifications, psychomotor or mental retardation, and severe brain damage [3].

Despite these tragic implications, the current therapy has not changed in the past few decades. Sulfonamides and pyrimethamine are the two drugs widely used to treat toxoplasmosis in humans. Although these drugs are helpful

Abbreviations: FBS, fetal bovine serum; HPMC, hydroxypropylmethylcellulose; MTT, microculture tetrazolium test; PBS, phosphate buffered saline

* Corresponding author. Tel.: +1 205 934 1132; fax: +1 205 934 8240.
E-mail address: m.elkouni@ccc.uab.edu (M.H. el Kouni).

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in the treatment of the acute stage of the disease, they usually do not eradicate infection and as many as 50% of the patients do not respond to therapy. The combination of sulfonamides and pyrimethamine is also ineffective against toxoplasma tissue cysts. In addition, prolonged exposure to this regimen induces serious host toxicity such as bone marrow suppression and severe skin rashes forcing the discontinuation of the therapy [2–5]. Other therapies, e.g. clindamycin or atovaquone, were met with limited success particularly in the long-term management of these patients. Furthermore, there is no effective vaccine currently available for the treatment of toxoplasmosis. Hence, there is a critical need to develop new and effective drugs with significant low host toxicity for the treatment and long term management of toxoplasmosis.

The rational drug design is usually based on biochemical and physiological differences between the pathogen and the host. One potential target for chemotherapeutic intervention against *T. gondii* is purine metabolism [6,7]. These parasites replicate rapidly and require large amounts of purines for the synthesis of their nucleic acids and other vital components [5–7]. However, *T. gondii*, in contrast to their host, are purine auxotrophs and hence must rely on the salvage of their purine requirements from the host ([7] and references therein).

Another striking difference between toxoplasma and their host is the nature of adenosine salvage. Adenosine is preferentially incorporated into the parasites nucleotide pools by at least a 10-fold higher rate than any other purine nucleobase or nucleoside tested [6,8]. Furthermore, in *T. gondii*, adenosine is directly phosphorylated to AMP, from which all other purine nucleotides can be synthesized to fulfill the parasite purine requirements. This reaction is catalyzed by the enzyme adenosine kinase (EC.2.7.1.20), which is almost 10 times more active than any other purine salvage enzyme in this parasite [6]. This contrasts sharply with most mammalian cells where adenosine is predominantly deaminated by adenosine deaminase (EC 3.5.4.4) to inosine, which is then cleaved by purine nucleoside phosphorylase (EC 2.4.2.1) to hypoxanthine as reviewed recently [7,9]. Neither of these two enzymes have any appreciable activity in *T. gondii* [6].

Structure-activity relationship studies as well as biochemical, metabolic and molecular investigations [7,9–14] demonstrated that the substrate specificity as well as other characteristics of *T. gondii* adenosine kinase differ significantly from those of the human enzyme and established the enzyme as an excellent potential chemotherapeutic target for the treatment of toxoplasmosis [7,9,11,14]. It was also demonstrated that 6-benzylthioinosine, among other 6-substituted purine nucleoside analogues, is a substrate for the parasite, but not the human adenosine kinase [7,9,11,14]. Furthermore, 6-benzylthioinosine was shown to be preferentially metabolized to the nucleotide level and becomes selectively toxic to *T. gondii* but not their host, thereby acting as a subversive substrate [7,9,10,14]. Therefore, modification of the chemical structure of 6-benzylthioinosine could further potentiate its anti-toxoplasmic efficacy. Herein, we report the testing of newly synthesized 6-benzylthioinosine analogues with various substitutions at their phenyl ring [14] as selective subversive substrates for *T. gondii* adenosine kinase and their efficacy as antitoxoplasmic agents in cell culture and in animal models.

2. Materials and methods

2.1. Chemicals

The 6-benzylthioinosine (6-benzyl 9-β-D-ribofuranosylpurine) analogues were synthesized as previously described [14]. The chemical structures of these compounds are shown in Table 1. [8-^{14}C]Adenosine (55 Ci/mol) and [5,6-^{13}H]uracil were purchased from Moravek Biochemicals. RPMI-1640 medium from GIBCO BRL; penicillin G and streptomycin sulfate from Mediatech/Cellgro; fetal bovine serum (FBS) from HyClone Laboratories. Silica Gel G thin layer chromatography (TLC) polygram plates were obtained from Fisher Scientific. Bovine γ-globulin and dye reagent for protein assays were from Bio-Rad Lab. Bugbustar was from Novagen. Hydroxypropylmethylcellulose (HPMC) was purchased from Sigma Chemical Company. All other chemicals and compounds were obtained from Sigma Chemical Co. or Fisher Scientific.

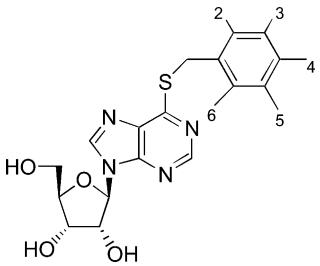
2.2. Maintenance of T. gondii

The RH and $TgAK^{-3}$ strains of *T. gondii* were propagated by intraperitoneal passage in female CD 1 mice (20–25 g). RH is a wild type strain and $TgAK^{-3}$ is a knockout mutant deficient in adenosine kinase [12]. Mice were injected i.p. with an inoculum (10^6 cells) of *T. gondii* contained in 0.2 mL of sterile PBS (phosphate buffered saline), pH 7.2, and were sacrificed after 2–3 days by inhalation of ether. The parasites were harvested from the peritoneal cavity by injection, aspiration and reinjection of 3–5 mL of PBS (two to three 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 produce parasite preparations that contain very little contamination and have a viability of >95%.

2.3. Preparation of parasites

When *T. gondii* were used for in vitro incorporation studies, the procedure was performed aseptically and the parasites were washed and resuspended in RPMI-1640 medium containing 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 3% fetal bovine serum.

Table 1
Chemical structures of 6-benzylthioinosine and its substituted analogues



Compound	2	3	4	5	6
1. 6-Benzylthioinosine	H	H	H	H	H
2. *o*-Fluoro-6-benzylthioinosine	F	H	H	H	H
3. *o*-Chloro-6-benzylthioinosine	Cl	H	H	H	H
4. *o*-Methyl-6-benzylthioinosine	CH_3	H	H	H	H
5. *m*-Nitro-6-benzylthioinosine	H	NO_2	H	H	H
6. *m*-Methyl-6-benzylthioinosine	H	CH_3	H	H	H
7. *m*-Trifluoromethyl-6-benzylthioinosine	H	CF_3	H	H	H
8. *p*-Fluoro-6-benzylthioinosine	H	H	F	H	H
9. *p*-Chloro-6-benzylthioinosine	H	H	Cl	H	H
10. *p*-Bromo-6-benzylthioinosine	H	H	Br	H	H
11. *p*-Cyano-6-benzylthioinosine	H	H	CN	H	H
12. *p*-Nitro-6-benzylthioinosine	H	H	NO_2	H	H
13. *p*-Methyl-6-benzylthioinosine	H	H	CH_3	H	H
14. *p*-Methoxy-6-benzylthioinosine	H	H	OCH_3	H	H
15. *p*-Trifluoromethoxy-6-benzylthioinosine	H	H	OCF_3	H	H
16. *p*-*tert*-Butyl-6-benzylthioinosine	H	H	*tert*-Butyl	H	H
17. *p*-Acetoxy-6-benzylthioinosine	H	H	$COOCH_3$	H	H
18. 2,4-Dichloro-6-benzylthioinosine	Cl	H	Cl	H	H
19. 3,4-Dichloro-6-benzylthioinosine	H	Cl	Cl	H	H
20. 2-Chloro-6-fluoro-6-benzylthioinosine	Cl	H	H	H	F
21. 2,4,6-Trimethyl-6-benzylthioinosine	CH_3	H	CH_3	H	CH_3

2.4. Overexpression of T. gondii adenosine kinase

The coding sequence of cloned *T. gondii* adenosine kinase gene *TgAK* [12] was PCR amplified and engineered into *Escherichia coli* expression vector pET21a at the *Ned*I/*Sac*I sites. The coding sequence was verified by DNA sequencing. The resulting plasmid pETgAK was transformed into strain BL21(DE3)plysS competent *E. coli* cells. For the expression of adenosine kinase, cells were grown at 37 °C in LB medium (pH 7.5) containing 50 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM and the incubation was continued for 3 h. The cells were then harvested by centrifugation at 6500 × *g* for 15 min at 4 °C. The cells were resuspended in 250 mL of cold 20 mM Tris–Cl pH 8.0, followed by centrifugation. The supernatant was removed and the pellet was stored at −70 °C. The frozen pellet was resuspended in 1 gm of bacterial paste in 5 mL of lysis buffer (20 mM Tris–Cl, pH 8, containing 1 mM AEBSF protease inhibitor, 10 μM leupeptin, 25 units/mL rBezonase, and 1× Bugbustar) and incubated at room temperature for 20 min. The suspension was then centrifuged at 100,000 × *g* for 30 min at 4 °C. The total protein concentration of the supernatant was determined and glycerol was added to a final concentration of 20% and stored at −20 °C.

2.5. Purification of T. gondii recombinant adenosine kinase

The purification of *T. gondii* adenosine kinase was performed using a computer controlled Pharmacia AKTA FPLC 900. The enzyme was purified to apparent homogeneity with a specific activity of 0.9 mmol/min/mg protein and a yield of 5 mg of soluble protein/L of culture by the following method. Aliquots of supernatant collected above were loaded onto a 220 mL Q-Sepharose Fast Flow column (Pharmacia) equilibrated with buffer A (20 mM Tris–Cl, pH 8.0 containing 15 mM β-mercaptoethanol). The unbound protein was washed with two column volumes of buffer A. The enzyme was then eluted with two column volumes with buffer A containing 100 mM NaCl. Fractions of 15 mL were collected. Adenosine kinase eluted with a broad peak at approximately 90–100 mM NaCl. The fractions containing the activity were pooled, dialyzed overnight in buffer A, concentrated, resuspended in buffer A, and reloaded on a 26 mL Q-Sepharose HR26/10 column (Pharmacia) equilibrated in

the same buffer. The column was first washed with 2 column volumes of buffer A. The protein was then eluted with 20 column volumes of liner gradient of 0–150 mM NaCl in buffer A, and 10 mL fractions were collected. Adenosine kinase eluted with a sharp peak at approximately 100 mM NaCl. Fractions containing the activity were pooled, concentrated, resuspended in buffer A. The chromatography step on the 26 mL Q-Sepharose HR26/10 column was repeated for a second time after which fractions containing the activity were pooled, concentrated, resuspended in buffer A and kept at −75 °C. The identity and homogeneity of the purified enzyme was established by mass spectrophotometry and SDS polyacrylamide gel electrophoresis as a single band at ∼49 KD, respectively. The enzyme is stable under these conditions for months.

2.6. Evaluation of 6-benzylthioinosine analogues as alternative substrates for purified T. gondii adenosine kinase

Enzyme assays were run under conditions where activity was linear with time and enzyme concentration [9,11]. Adenosine kinase activity was determined by following the formation of radiolabeled AMP from adenosine. The assay mixture contains 50 mM Tris–Cl, pH 7.4, 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM creatine phosphate, creatine kinase, 5 μM [8-^{14}C]adenosine (55 Ci/mol), 50 μL of the purified recombinant enzyme in a final volume of 100 μL, in the absence or presence of various concentrations of the compound under evaluation. Incubation was carried out at 37 °C and terminated by boiling in a water bath for 2 min followed by freezing for at least 20 min. Precipitated proteins were removed by centrifugation and 10 μL of the supernatant was spotted on silica gel TLC plates. The TLC plates were developed in a mixture of chloroform/methanol/acetic acid (102:12:6, v/v/v). The R_f values were: adenosine, 0.27; AMP, 0.17. The amounts of radioactivity in both the substrate and product(s) were calculated on a percentage basis using a computerized Berthold LB-284 Automatic TLC-Linear Analyzers (Wallac Inc.). Apparent K_i values of these analogues were calculated from Dixon plots $1/v$ versus $[I]$ by least-squares fitting by computer programs developed by Dr. F.N.M. Naguib as previously described [9,11].

2.7. HPLC analysis of the products of the enzyme assays

The products and substrates of the above described enzyme assays were also separated by HPLC. For these studies, Hewlett Packard 1050 HPLC systems equipped with autosamplers, autoinjectors, quaternary pumps, multiple wave length Diode array base triple channel UV monitors, and on line Packard radioactive flow detectors are used. The systems are operated by computer programs which handle data analysis, comparison, and storage of data after each run. Nucleoside and nucleotide contents are analyzed using a BioBasic AX (25 cm × 0.46 cm, ODS 5 μm) column (ThermoHypersil-Keystone). A 100 μL sample is injected. Elution is carried out for 45 min with a flow rate of 1 mL/min starting with a 15 min linear gradient of buffer A [5 mM ammonium acetate, 0.5% acetonitrile (pH 8.0)], to buffer B [50 mM ammonium acetate, 75% acetonitrile (pH 8.0)]. This was followed by a 10 min runtime of 100% solution A, then followed by a 20 min runtime of 100% buffer C (HPLC grade methanol). The eluent is monitored at 254 nm and λ_{max} of the compound under study. Compounds are identified by retention time, coelution with authentic samples and/or absorbance ratio of the compound ($s\lambda_{max}$/254 nm. The retention times of AMP, ADP and ATP were 20, 28, and 33 min, respectively. Mass spectrometry analyses were used to verify the identity of the products as described below.

2.8. Mass spectrometry analyses

Mass spectrometry analyses were performed on a Micromass Q-Tof 2 mass spectrometer (Micromass). The samples were dissolved in 50/50 acetonitrile/water containing 0.1% formic acid and injected into a 1 μL/min flow of the same solvent. The flow was introduced into the nano-lc interface of the mass spectrometer. The mass spectra were recorded in the negative ion mode.

2.9. Protein determination

Protein concentrations were determined by the Bradford method [15] using the Bio-Rad Laboratories protein assay kit and bovine γ-globulin as a standard.

2.10. Evaluation 6-benzylthioinosine and its analogues as potential anti-toxoplasmosis agents against T. gondii in tissue culture

The wild type RH and the adenosine kinase deficient mutant $TgAK^{-3}$ [12] strains of *T. gondii* were used in these experiments. The adenosine kinase deficient mutant $TgAK^{-3}$ was used as a control to verify that the promising drugs were metabolized by adenosine kinase in vivo. The effects of purine analogues as anti-toxoplasmosis agents in tissue culture was measured by their ability to inhibit the replication of intracellular *T. gondii* in tissue culture using monolayers of human foreskin fibroblasts (CRL-1634, American Type Culture Collection) grown for no more than 20 passages in RPMI 1640 medium [10,14]. The viability of intracellular parasites was evaluated by the selective incorporation of radiolabeled uracil into nucleic acids of the parasites at least in triplicates as previously described [7,9,10,14]. Briefly, confluent cells (4–5-day incubation) were cultured for 24 h in the 24-well flat bottom microtiter plates (∼5 × 10^5/1 mL/well) and incubated at 37 °C in 5% CO_2, 95% air to allow the cells to

attach. The medium was then removed and the cells were infected with isolated *T. gondii* in medium with 3% FBS (1 parasite/cell). After 1 h incubation, the cultures were washed with media with 10% FBS to remove extracellular parasites. FBS was maintained at a final concentration of 10%. Compounds were dissolved in 50% ethanol and then added to cultures of the parasite-infected cells to give a final concentration of 0, 5, 10, 25, and 50 μM. The final concentration of ethanol when the compounds were added to the wells was 2.5%. After an additional 18 h incubation the medium was replaced with 1 mL drug free media containing [5,6-^{13}H]uracil (5 μCi/mL) and incubated for another 6 h after which the media was removed. The fibroblasts were then released from the wells by trypsinization with the addition of 200 μL trypsin/EDTA (2.5×) to each well. After 10 min incubation, 1 mL of ice cold 10% trichloroacetic acid (TCA) was added to each well. The plates were then placed on a shaker to insure the detachment of the cells. The suspended contents of each well was filtered through GF/A 2.4 cm glass microfiber filters (Whatman), which were prewashed each with 1 mL double distilled H_2O and dried. After filtration, the filters were washed with 10 mL methanol, left to dry, then placed in scintillation vials containing 5 mL of Econo-Safe scintillation fluor (Research Products International Corp., Mount Prospect, IL), and radioactivity was counted using an LS5801 Beckman scintillation counter. The effect of the compounds on the growth of the parasite was estimated as percentage reduction in the uptake of radiolabeled uracil by treated parasites as compared to the untreated controls [7,9,18]. Radiolabel incorporation closely correlates with parasite growth [7,9,18]. The IC_{50} (the concentration causes 50% inhibition) values were calculated from the growth inhibition data by Win NonLin version 3.2 (Pharsight Corporation).

2.11. Host-toxicity of 6-benzylthioinosine and its analogues

Possible toxicity against the host cells by the same doses of the various analogues used in the above experiments was measured, at least in triplicates, using a modification of the microculture tetrazolium (MTT) assay on uninfected monolayers of human foreskin fibroblasts (grown for no more than 20 passages) in RPMI 1640 medium [10,14]. Briefly, confluent cells were incubated for at least 24 h in 96-well flat bottom microtiterplates (~10^5/200 μL/well) at 37 °C in 5% CO_2, 95% air to allow the cells to attach. The medium was then replaced with 200 μL of fresh medium. The appropriate concentration of the compounds was dissolved in 50 μL of medium, and added to each well to give the desired final concentrations. The cultures were then incubated for 48 h after which 50 μL of sterile MTT solution (2 mg/1 mL PBS) was added to each well. MTT solution was sterilized by filtration through 0.22 μm filters (Costar, Cambridge, MA). After 4 h incubation, the medium was removed and 100 μL of dimethylsulfoxide (DMSO) was added to each well and the plates were shaken gently for 2–3 min to dissolve the formed formazan crystals. The absorbance was measured at 540 nm using a computerized microtiterplate reader (Themomax, Molecular Devices).

2.12. Evaluation of anti-toxoplasmic activity in mice

Preliminary studies of the efficacy of 6-benzylthioinosine and its analogues as antitoxoplasmic agents were performed in female CD-1 mice (20–25 g) obtained from Charles River Laboratories (Wilmington, MA) Mice were housed 5 mice/cage with water and food ad libitum under a normal light cycle (light, 06:00–18:00 h; dark, 18:00–06:00 h) according to the guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Each mouse was infected with 200 tachyzoites (0.2 mL of toxoplasma suspension in PBS containing 0.25 parasite/field under 40× magnification of a light microscope). The compounds were administered orally every 8 h for 5 days. The drugs were mixed well with HPMC powder in hot water (80 °C) and homogenized thoroughly using a polytron homogenizer (Brinkmann Instruments). The final concentration of HPMC was 0.75%. The drug solution was vortexed well before and periodically during dosing. HPMC was preferred over the commonly used methylcellulose because the latter must be cooled to (10 °C) in order to hydrate completely [16]. Drugs were administered orally (0.1 mL/10 g) using 18G intubation needles (Popper and Sons Inc.). Control mice received the carrier solution (0.75% HPMC).

3. Results and discussion

3.1. Binding affinities

6-Benzylthioinosine and its newly synthesized analogues (Table 1) were tested as ligands of human and *T. gondii* adenosine kinases. None of these compounds bound to the human hepatic enzyme at the concentrations tested. On the other hand, 6-benzylthioinosine and all the analogues tested bound to *T. gondii* adenosine kinase, albeit to different degrees. The binding affinities (apparent K_i values) of these compounds with *T. gondii* adenosine kinase are shown in Table 2. The results in Table 2 show that several 6-benzylthioinosine analogues bound better to *T. gondii* adenosine kinase than 6-benzylthioinosine. *p*-Cyano-6-benzylthioinosine (**11**) and 2,4-dichloro-6-benzylthioinosine (**18**) were the best ligands. *p*-Trifluoromethoxy-6-benzylthioinosine (**15**) and 2,4,6-trimethyl-6-benzylthioinosine (**21**) were the weakest ligands. In addition, HPLC analysis of the substrates and products of the enzyme assays demonstrated that 6-benzylthioinosine (**1**) and its analogues were converted to their respective

Table 2
Binding affinity (apparent K_i) of 6-benzylthioinosine and analogues to *Toxoplasma gondii* adenosine kinase and the effect of different doses on host-toxicity and percent survival of wild type and adenosine kinase deficient ($TgAK^{-3}$) strains of the parasite grown in human fibroblasts in culture

Compound	K_i (μM)[a]	Infection	Concentration (μM)					IC_{50} (μM)
			0	5	10	25	50	
1. 6-Benzylthioinosine	2.4 ± 1.2	Wild type	100	62.3	53.7	23.8	14.8	9.3 ± 4.3
		$TgAK^{-3}$	100	99.7	99.3	100	100	
		None	100	99.9	99.8	99.1	99.1	
2. *o*-Fluoro-6-benzylthioinosine	*[b]	Wild type	100	67.1	45.5	9.6	0.0	8.2 ± 5.1
		$TgAK^{-3}$	100	91.4	99.7	98.3	100	
		None	100	93.7	99.8	100	99.5	
3. *o*-Chloro-6-benzylthioinosine	1.4 ± 0.2	Wild type	100	72.6	27.1	8.3	0.0	6.7 ± 4.1
		$TgAK^{-3}$	100	98.8	100	100	100	
		None	100	97.0	97.8	99.7	99.8	
4. *o*-Methyl-6-benzylthioinosine	1.5 ± 0.6	Wild type	100	78.9	37.2	16.8	0.0	7.7 ± 4.3
		$TgAK^{-3}$	100	100	100	100	100	
		None	100	100	95.41	100	100	
5. *m*-Nitro-6-benzylthioinosine	3.0 ± 1.6	Wild type	100	60.9	29.9	0.8	0.0	6.2 ± 4.2
		$TgAK^{-3}$	100	100	97.0	91.8	94.6	
		None	100	94.8	95.3	99.2	93.3	
6. *m*-Methyl-6-benzylthioinosine	1.3 ± 0.6	Wild type	100	84.1	32.1	6.7	0.0	8.2 ± 7.0
		$TgAK^{-3}$	100	100	100	100	100	
		None	100	98.4	100	100	100	
7. *m*-Trifluoromethyl-6-benzylthioinosine	2.9 ± 1.3	Wild type	100	80.2	36.8	4.7	0.0	8.7 ± 7.4
		$TgAK^{-3}$	100	100	100	100	100	
		None	100.	99.0	95.7	94.9	96.9	
8. *p*-Fluoro-6-benzylthioinosine	1.2 ± 0.3	Wild type	100	85.0	46.3	26.6	0.6	10.3 ± 7.6
		$TgAK^{-3}$	100	100	100	100	100	
		None	100	95.5	91.2	97.7	95.0	
9. *p*-Chloro-6-benzylthioinosine	2.1 ± 1.0	Wild type	100	83.5	50.9	22.7	0.0	8.11 ± 5.0
		$TgAK^{-3}$	100	100	100	97.89	100	
		None	100	100	98.1	99.0	98.1	
10. *p*-Bromo-6-benzylthioinosine	7.5 ± 2.6	Wild type	100	94.3	72.0	30.4	0.5	14.3 ± 12.9
		$TgAK^{-3}$	100	99.0	100	100	96.7	
		None	100	97.1	100	100	99.5	
11. *p*-Cyano-6-benzylthioinosine	0.9 ± 0.1	Wild type	100	61.5	45.2	7.6	0.0	4.3 ± 2.1
		$TgAK^{-3}$	100	100	100	100	100	
		None	100	97.4	96.9	100	99.7	
12. *p*-Nitro-6-benzylthioinosine	1.1 ± 0.2	Wild type	100	86.3	51.9	33.5	0.5	12.0 ± 9.3
		$TgAK^{-3}$	100	100	100	100	100	
		None	100	96.7	94.4	96.3	96.6	
13. *p*-Methyl-6-benzylthioinosine	3.3 ± 1.1	Wild type	100	75.1	37.1	5.5	0.0	7.8 ± 5.1
		$TgAK^{-3}$	100	100	100	99.0	100	
		None	100	98.1	94.1	92.9	98.3	
14. *p*-Methoxy-6-benzylthioinosine	2.6 ± 0.4	Wild type	100	51.3	20.1	0.2	0.0	3.5 ± 1.7
		$TgAK^{-3}$	100	98.4	100	100	98.1	
		None	100	99.8	100	100	89.9	
15. *p*-Trifluoromethoxy-6-benzylthioinosine	141 ± 8.3	Wild type	100	93.1	64.9	54.5	44.0	24.8 ± 13.8
		$TgAK^{-3}$	100	100	100	91.6	95.1	
		None	100	99.9	97.4	98.9	98.6	
16. *p-tert*-Butyl-6-benzylthioinosine	113 ± 11.7	Wild type	100	83.3	71.7	47.2	12.2	23.3 ± 10.8
		$TgAK^{-3}$	100	100	100	99.9	100	
		None	100	99.7	100	99.1	95.1	
17. *p*-Acetoxy-6-benzylthioinosine	96.1 ± 1.6	Wild type	100	88.4	53.0	52.1	26.2	15.0 ± 6.5
		$TgAK^{-3}$	100	100	99.3	100	100	
		None	100	99.3	99.8	97.3	99.3	
18. 2,4-Dichloro-6-benzylthioinosine	0.7 ± 0.2	Wild type	100	94.8	79.1	34.5	0.0	7.3 ± 1.8
		$TgAK^{-3}$	100	100	98.9	100	99.2	
		None	100	98.1	99.2	94.0	99.5	

Table 2 (*Continued*)

Compound	K_i (μM)[a]	Infection	Concentration (μM)					IC_{50} (μM)
			0	5	10	25	50	
19. 3,4-Dichloro-6-benzylthioinosine	2.5 ± 1.3	Wild type	100	75.5	66.3	6.04	0.0	10.4 ± 6.35
		TgAK^{-3}	100	100	100	100	100	
		None	100	95.4	97.8	90.2	90.4	
20. 2-Chloro-6-fluoro-6-benzylthioinosine	37.7 ± 2.3	Wild type	100	72.7	45.0	4.83	0.0	8.7 ± 6.5
		TgAK^{-3}	100	100	100	88.0	100	
		None	100	98.2	95.8	86.4	85.9	
21. 2,4,6-Trimethyl-6-benzylthioinosine	150 ± 2.7	Wild type	100	94.1	71.9	59.2	21.9	31.1 ± 9.1
		TgAK^{-3}	100	100	99.9	100	100	
		None	100	99.5	100	100	98.4	
Sulfadiazine		Wild type	100	75.7	64.5	59.2	46.7	7.0 ± 1.9
		None	100	98.2	99.7	99.8	102.5	
Pyrimethamine		Wild type	100	53.8	36.3	11.8	6.9	5.3 ± 1.0
		None	100	103.6	98.8	108.2	118.4	

Percent survival of parasites was measured by incorporation of [5,6-^{3}H]uracil. Host toxicity of uninfected cells was measured by MTT method described in Section 2.

[a] Data from ref. [14].

[b] Could not be determined.

nucleotides by *T. gondii* adenosine kinase. Fig. 1 shows the reversed-phase HPLC profile of the metabolism of *p*-cyano-6-benzylthioinosine (**11**) to its 5′-monophosphate. The other 6-substituted analogues have similar metabolic profiles although the retention time and amount of the nucleotides synthesized differed between the various compounds. The chemical structures of the respective nucleoside 5′-monophosphates of the compounds under study were verified by mass spectrometry. Fig. 2 shows, as an example, the mass spectrum of *p*-cyano-6-benzylthioinosine (**11**) 5′-monophosphate. The metabolism of 6-benzylthioinosine (**1**) and its analogues, to their respective nucleoside 5′-monophosphates indicates that these compounds are alternate substrates of *T. gondii* adenosine kinase. Therefore, their apparent K_i values are equal to their apparent K_m values [17].

The data in Table 2 demonstrated that there was no significant differences in the binding affinities between analogues with electron-rich (CH_3, **4**) or electron-deficient (Cl, **3**) substitutions at either the *ortho* or *meta* position on the phenyl ring of the benzyl group. On the other hand, substitution at the *para* position seemed to be affected by both the hydrophobicity and the electronegativity of the substituent. In general, electron-deficient substituents, e.g. F (**8**), Cl (**9**), CN (**11**) and NO_2 (**12**), were preferred. In the case of halogens, the increase in the electronegativity of the halogen, F (**8**) 6 Cl (**9**) 6 Br (**10**) was accompanied by increased binding to the enzyme. The size of the substitution at the *para* position also appeared to play a critical role in binding. Increasing the bulkiness of substitution, e.g. acetoxy (**16**) and *tert*-butyl (**17**), decreased the binding drastically.

In the case of multiple substitutions, it was noted that substitutions at both the *ortho* and *para* positions, e.g. 2,4-dichloro-6-benzylthioinosine (**18**), resulted in better binding than when both the same substitutions were at the *meta* and *para* positions, e.g. 3,4-dichloro-6-benzylthioinosine (**19**). Triple substitutions, e.g. 2,4,6-trimethyl analogue (**21**) showed drastic loss of binding, whereas a single methyl substitution at the *ortho* (**4**), *meta* (**6**) or *para* (**13**) positions exhibited appreciable binding.

Previous structure activity relationships [11,14] and crystal structures studies of *T. gondii* adenosine kinase [13] demonstrated that two hydrophobic pockets are present on the *T. gondii* adenosine kinase, one at the 6-position and another at the 7-position of the purine ring. The hydrophobic pocket at the 6-position is larger than that at 7-position. Furthermore, docking calculations [14] showed that the benzyl group of 6-benzylthioinosine occupied the 6-position hydrophobic pocket in a favorable manner. The overall orientation of the benzyl group was always in the same region in spite of the fact that conformational changes occur at the glycosidic bond or the pentose ring [14]. This binding pattern was not affected by different substitution on the phenyl ring of the benzyl group. Furthermore, the hydrophobic interactions inside this pocket were enhanced by increasing the hydrophobicity or electronegativity of the substitution on the phenyl ring [14]. Such effect was more pronounced at the *para* position than at the *ortho* position. The combined effect of the hydrophobic and electronegative potential on the benzyl group can explain the better binding of *p*-cyano-6-benzylthioinosine (**11**) and 2,4-dichloro-6-benzylthioinosine (**18**). The presence of cyano group at the *para* position of *p*-cyano-6-benzylthioinosine (**11**) increased the van der Waals interactions with the surrounding residues while the benzyl group positions itself in the hydrophobic pocket [14]. The observed enhancement of the binding affinity of 2,4-dichloro-6-benzylthioinosine (**18**) over that of *p*-cyano-6-benzylthioinosine (**11**) could be due to the

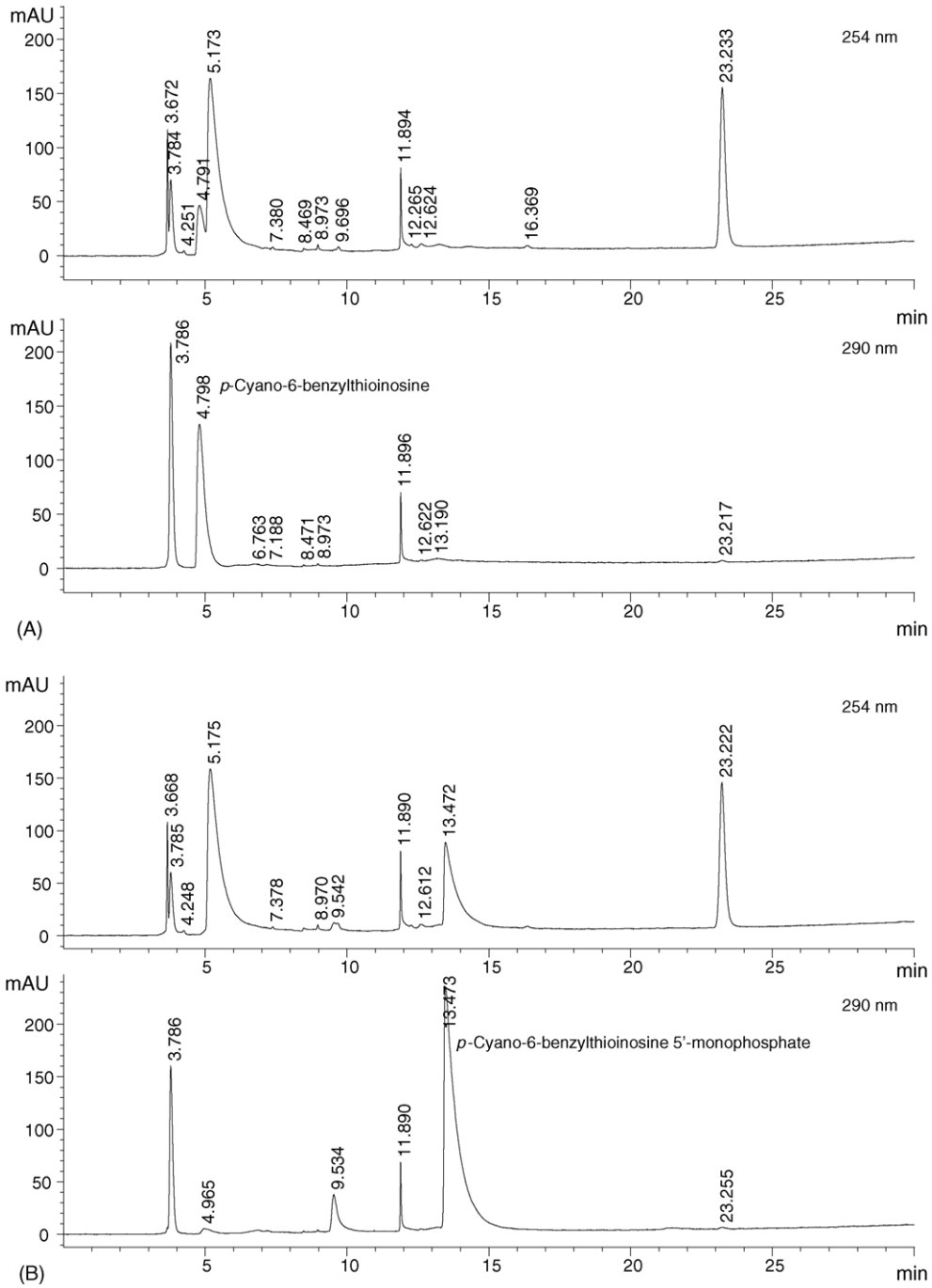


Fig. 1. The reversed-phase HPLC profile of the metabolism of *p*-cyano-6-benzylthioinosine to its 5′-monophosphate. (A) Controls, *p*-cyano-6-benzylthioinosine in reaction mixture after incubation without the toxoplasma adenosine kinase. (B) Experimental, *p*-cyano-6-benzylthioinosine after incubation with the toxoplasma adenosine kinase. In both profiles (A) and (B), the above panel shows the profile monitored at 254 nm and the lower panel shows the profile monitored at 290 nm, the λ_{max} of *p*-cyano-6-benzylthioinosine.

increase in the net electronegative potential on the benzyl ring of 2,4-dichloro-6-benzylthioinosine (**18**). On the other hand, the decrease in the binding affinities of analogues with bulky substitutions on the phenyl ring as in the case with *p*-trifluoromethoxy-6-benzylthioinosine (**15**), *p*-*tert*-butyl-6-benzylthioinosine (**16**) and *p*-acetoxy-6-benzylthioinosine (**17**) could be caused by steric hindrance. It was shown that the hydrophobic pocket around the 6-position of the purine ring has a long funnel-like shape with its broader end near the 6-position of the purine ring [14]. The funnel-like shape of the pocket can accommodate the phenyl moiety with small substituents, like halogens, which are electronegative and can form favorable van der Waals interactions with the lining hydrogen atoms of the surrounding residues [14]. However, increasing the size of such substituents as in the case with *p*-trifluoromethoxy-6-benzylthioinosine (**15**), *p*-*tert*-butyl-6-benzylthioinosine (**16**) and *p*-acetoxy-6-benzylthioinosine

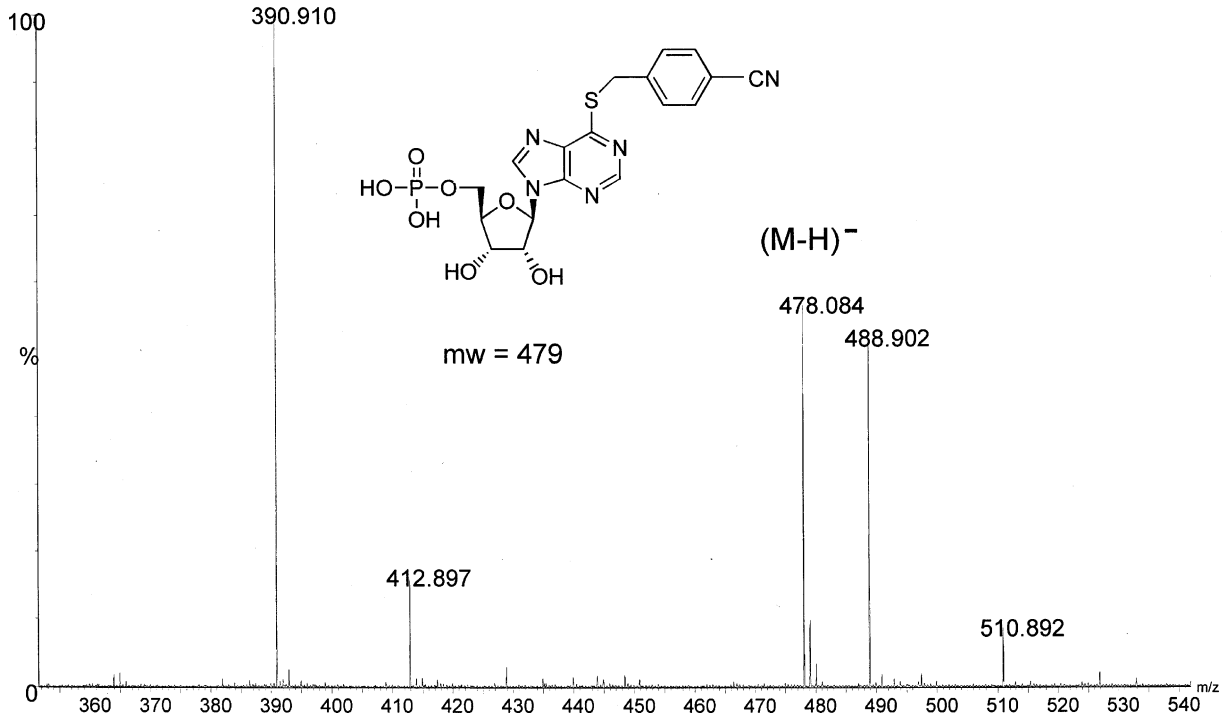


Fig. 2. Negative electrospray mass spectrum of *p*-cyano-6-benzylthioinosine 5′-monophosphate showing the ($M - H$) ion at 478. Residual phosphoric acid clusters are seen at 390.9 and 488.9.

(**17**) can cause steric interference with the side chains of the residues lining the pocket. Finally, the funnel-like structure of the pocket could be the reason why substituents that are tolerated at the *para* position can be less tolerated at the *meta* position.

3.2. Evaluation of anti-toxoplasmic activity and host-toxicity in tissue culture

6-Benzylthioinosine and its analogues were evaluated as potential anti-toxoplasmic agents against wild type (RH) and adenosine kinase deficient ($TgAK^{-3}$) strains of *T. gondii* grown in human foreskin fibroblasts in culture. As a positive control, pyrimethamine and sulfadiazine, the standard chemotherapeutic agents used in the treatment of toxoplasmosis, were also evaluated. The inhibitory effects of these compounds on the growth of toxoplasma were measured using uracil uptake assays. Uracil uptake assays are highly specific to *T. gondii* as mammalian cells do not incorporate uracil into their nucleoside and nucleotide pool or nucleic acids [7,9,18]. Therefore, an exponential increase in radiolabel incorporation closely correlates with the exponential growth of the parasite [7,9,18]. Using this method, all the tested compounds were effective, albeit to different degrees, against infection with the wild type parasites and the inhibition was dose dependent (Table 2). *p*-Cyano-6-benzylthioinosine (**11**) and *p*-methoxy-6-benzylthioinosine (**14**) were better than the parent compound (**1**) and were the most effective antitoxoplasmic agents. In a striking contrast, none of these compounds was effective against infection with the adenosine kinase deficient strain $TgAK^{-3}$. The lack of sensitivity of $TgAK^{-3}$ to these purine nucleoside analogues could not be due to a lower growth rate of the mutant. $TgAK^{-3}$ is much more aggressive in its growth than the RH wild type in both tissue culture and animals (unpublished results). Therefore, it is concluded that the presence of *T. gondii* adenosine kinase is a requirement for 6-benzylthioinosine and its analogues to exert their anti-toxoplasmic effect. Consequently, these compounds are substrates for *T. gondii* adenosine kinase in vivo as was the case with the in vitro enzyme assays. However, the results in Table 2 show that there are discrepancies between the order of potency of the compounds as ligands to the enzyme (apparent K_i) and as chemotherapeutic agents (IC_{50}). These discrepancies in the order of potencies or lack of strong correlation between the apparent K_i and IC_{50} values could be attributed to differences in cellular uptake of these compounds. Nevertheless, there was a general trend that compounds with large apparent K_i values, e.g. *p*-trifluoromethoxy-6-benzylthioinosine (**15**) and 2,4,6-trimethyl-6-benzylthioinosine (**21**) were less potent and have larger IC_{50} values than compounds with small apparent K_i values, e.g. cyano-6-benzylthioinosine (**11**) and *p*-methoxy-6-benzylthioinosine (**14**).

Table 2 also shows that all compounds had no toxic-side-effects on the survival of uninfected host cell indicating that host toxicity is of little concern for these compounds. The lack of host toxicity is due to at least two factors. First, such 6-substituted compounds are not substrates for the human adenosine kinase (the present results and [7,9,10]).

Thus, no toxic nucleotides were formed in the absence of the parasite enzyme. Secondly, the newly synthesized compounds are analogues of *p*-nitrobenzylthioinosine (NBMPR), a known inhibitor of nucleoside transport in mammalian cells. Therefore, the newly synthesized compounds may act as NBMPR and not gain entry to uninfected host cells to exert its toxic effects. Indeed, our previous studies have demonstrated that NBMPR is not transported and/or metabolized by uninfected host cells or cells infected with mutant parasites lacking the adenosine/purine transporter [7,9,10]. On the other hand, NBMPR is transported and metabolized by cells infected with wild type toxoplasma [7,9,10]. Thus, the presence of a functional toxoplasma adenosine/purine transporter and adenosine kinase are prerequisites for the transport and metabolism of the 6-substituted compounds by infected cells [7,9,10]. Finally, the results in Table 2 show that some compounds (e.g. **11** and **14**) were more active than sulfadiazine and the effectiveness of compound **11** was comparable to that of pyrimethamine. Therefore, 6-benzylthioinosine and its analogues are selectively toxic to *T. gondii* infected cells and are promising selective therapeutic agents against toxoplasmosis.

3.3. Evaluation of anti-toxoplasmic activity in mice

In preliminary experiments, we tested the effect of 6-benzylthioinosine (**1**) and its analogues, *p*-cyano-6-benzylthioinosine (**11**) and 2,4-dichloro-6-benzylthioinosine (**18**) on mice infected with the virulent wild type RH strain of toxoplasma as well as on non-infected mice. The results in Table 3 show that all three compounds at 100 mg/kg increased the life span of infected mice from 6 to 8 days. There was no apparent advantage of one compound over the other in these preliminary tests. Since these are preliminary results, with only one dose, it is premature to asses the significance of the 2 days increase in life span nor can it be claimed that these compounds are better or worse than the clinically used anti-toxoplasmic drugs. Refining the animal model (e.g. different doses, schedules, appropriate toxoplasma strains other than the exceptionally virulent RH strain), after performing appropriate pharmacokinetic and toxicological studies, which is beyond the scope of the present investigation, would give a better assessment for these compounds as anti-toxoplasmic agents. However, it is clear that analogues of 6-benzylthioinosine have anti-toxoplasmic effect and they work by a different mechanism than the other known anti-toxoplasmic drugs. Hence, these compounds are promising prototypes of antitoxoplasmic agents which can be used alone or in combination with other existing drugs. It is encouraging that the doses used did not show any apparent host-toxicity.

Table 3
Effect of 6-benzylthioinosine and analogues on the survival of mice infected with the RH strain of *T. gondii*

Treatment	Infection	Survival			
		Day 5	Day 6	Day 7	Day 8
None	Yes	3/5	0/5		
6-Benzylthioinosine (**1**)	Yes	5/5	5/5	5/5	0/5
	No	5/5	5/5	5/5	5/5
p-Cyano-6-benzylthioinosine (**11**)	Yes	5/5	5/5	5/5	1/5
	No	5/5	5/5	5/5	5/5
2,4-Dichloro-6-benzylthioinosine (**18**)	Yes	5/5	5/5	5/5	1/5
	No	5/5	5/5	5/5	5/5

Each mouse was infected with 200 tachyzoites and the treatment group received oral 100 mg/kg of the compound every 8 h for 5 days. Controls received the carrier solution.

The mechanism and/or targets of toxicities of 6-benzylthioinosine and its analogues are not known at the present time. However, such toxicities are clearly mediated by the nucleotides of these analogues since no toxicities were observed in the absence of adenosine kinase and lack of nucleotide synthesis. Among the possible targets that the nucleotides of 6-benzylthioinosine and its analogues could interfere with, are other steps involved in purine salvage, nucleic acids and/or protein synthesis as well as reactions carried out by protein kinases leading to the death of the parasite. Experiments are currently performed in our laboratories to address these questions.

In conclusion, we have demonstrated that 6-benzylthioinosine and its analogues are subversive substrates of *T. gondii*, but not the human, adenosine kinase. As a result, 6-benzylthioinosine and its analogues exhibited selective anti-toxoplasmic effect in cell culture and animal models. The binding affinity of these compounds to *T. gondii* adenosine kinase and their efficacy as selective antitoxoplasmic agents varied according to the nature and position of various substituents on the phenyl ring of the benzyl group. Therefore, further rationally designed modifications of these 6-substituted purine nucleosides could yield better selective subversive substrates of toxoplasma adenosine kinase and, hence more potent anti-toxoplasmosis agents.

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