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

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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-benzylthioinosines act as subversive substrates of *T. gondii*, but not human, adenosine kinase. Therefore, these compounds are preferentially metabolized to their respective nucleotides and become selectively toxic against the parasites but not their host. Moreover, 7-deazaadenosine (tubercidin) was shown to be an excellent ligand of *T. gondii* adenosine kinase. Therefore, we synthesized 7-deaza-6-benzylthioinosine, and analogues with various substitutions at their phenyl ring, to increase the binding affinity of the 6-benzylthioinosines to *T. gondii* adenosine kinase. Indeed, the 7-deaza-6-benzylthioinosine analogues were better ligands of *T. gondii* adenosine kinase than the parent compounds, 6-benzylthioinosine and 7-deazainosine. Herein, we report the testing of the metabolism of these newly synthesized 7-deaza-6-benzylthioinosines, as well as their efficacy as anti-toxoplasmic agents in cell culture. All the 7-deaza-6-benzylthioinosine analogues were metabolized to their 5′-monophosphate derivatives, albeit to different degrees. These results indicate that these compounds are not only ligands but also substrates of *T. gondii* adenosine kinase. All the 7-deaza-6-benzylthioinosine analogues showed a selective antitoxoplasmic effect against wild type parasites, but not mutants lacking adenosine kinase. The efficacy of these compounds varied with the position and nature of the substitution on their phenyl ring. Moreover, none of these analogues exhibited host toxicity. The best compounds were 7-deaza-6-(*p*-methoxybenzylthio)inosine (IC_{50} = 4.6 μM), 7-deaza-6-(*p*-methoxycarbonylbenzylthio)inosine (IC_{50} = 5.0 μM), and 7-deaza-6-(*p*-cyanobenzylthio)inosine (IC_{50} = 5.3 μM). These results further confirm that *T. gondii* adenosine kinase is an excellent target for chemotherapy and that 7-deaza-6-benzylthioinosines are potential antitoxoplasmic agents.

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Abbreviations: FBS, fetal bovine serum; MTT, microculture tetrazolium test; NBMPR, nitrobenzylthioinosine.

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1. Introduction

The parasitic protozoan, *Toxoplasma gondii*, is the etiologic agent for toxoplasmosis, a parasitic disease wide spread among various warm-blooded animals including man [1]. Approximately a billion people worldwide, including 60% of the population in the US, are seropositive to *T. gondii*. Infection with *T. gondii* is asymptomatic (90% of cases) in the general population. By contrast, the disease 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. The efficacy of the current therapy for toxoplasmosis (a combination of pyrimethamine and sulfadiazine) is limited, primarily by serious host toxicity and ineffectiveness against tissue cysts. Furthermore, as many as 50% of patients do not respond to therapy. 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, spiramycin or atovaquone, have met with limited success, particularly in the long-term management of these patients. 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.

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. These parasites replicate rapidly and require large amounts of purines for the synthesis of their nucleic acids and other vital components. In contrast to their host, however, *T. gondii* are purine auxotrophs and must rely on the salvage of their purine requirements from the host [6,7 and references therein].

Another striking difference between toxoplasma and their host is the nature of adenosine salvage. Adenosine is preferentially incorporated into the parasite nucleotide pool by at least a 10-fold higher rate than any other purine nucleobase or nucleoside tested [8,9]. Furthermore, 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 [8]. 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 previously reviewed [6,7]. Neither of these two enzymes have any appreciable activity in *T. gondii* [8].

Structure–activity relationships [10–12], biochemical [11,13,14], metabolic [6,13–16], and molecular [17] investigations have demonstrated that the substrate specificity, as well as other characteristics of *T. gondii* adenosine kinase, differs significantly from those of the human enzyme, and have established the enzyme as an excellent potential chemotherapeutic target for the treatment of toxoplasmosis [6,7]. It was also demonstrated that 6-benzylthioinosine, among other 6-substituted purine nucleoside analogues, is a substrate for the parasite, but not human adenosine kinase [6,11,13–15]. Furthermore, 6-benzylthioinosine was shown to be metabolized preferentially to the nucleotide level and becomes selectively toxic to *T. gondii*, but not their host, thereby acting as a subversive substrate [6,11,13–15]. Therefore, modification of the chemical structure of 6-benzylthioinosine could further potentiate its antitoxoplasmic efficacy.

Structure–activity relationships [10] also showed that 7-deazaadenosine is one of best ligands of *T. gondii* adenosine kinase. Therefore, we thought that elimination of the N7 of inosine would enhance the binding of 6-benzythioinosines to *T. gondii* adenosine kinase. Indeed, 7-deaza-6-benzylthioinosine analogues were better ligands than the parent compounds [12]. Herein, we report the testing of newly synthesized 7-deaza-6-benzylthioinosine analogues, with various substitutions at their phenyl ring [12], as subversive substrates for *T. gondii* adenosine kinase and their efficacy as antitoxoplasmic agents in cell culture.

2. Materials and methods

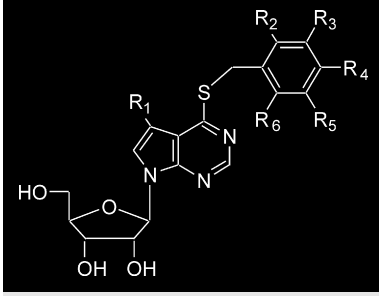
2.1. Chemicals

The 7-deaza-6-benzylthioinosine (1) analogues (**1–24**) were synthesized as previously described [12]. The chemical structures of these compounds are shown in Table 1. 7-Deazainosine and 6-benzylthioinosine were generously provided by Dr. Mohamed Nasr, Drug Development and Clinical Sciences Branch, NIAID. [5,6-^{13}H]uracil was 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. 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 [17]. 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

Table 1 – Chemical structure of 7-deaza-6-benzylthioinosine and its substituted analogue



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1. 7-Deaza-6-benzylthioinosine	–H	–H	–H	–H	–H	–H
2. 7-Deaza-6-(*o*-fluorobenzylthio)inosine	–H	–F	–H	–H	–H	–H
3. 7-Deaza-6-(*o*-chlorobenzylthio)inosine	–H	–Cl	–H	–H	–H	–H
4. 7-Deaza-6-(*o*-bromobenzylthio)inosine	–H	–Br	–H	–H	–H	–H
5. 7-Deaza-6-(*o*-methylbenzylthio)inosine	–H	$-CH_3$	–H	–H	–H	–H
6. 7-Deaza-6-(*m*-nitrobenzylthio)inosine	–H	–H	$-NO_2$	–H	–H	–H
7. 7-Deaza-6-(*m*-methylbenzylthio)inosine	–H	–H	$-CH_3$	–H	–H	–H
8. 7-Deaza-6-(*m*-trifluoromethylbenzylthio)inosine	–H	–H	$-CF_3$	–H	–H	–H
9. 7-Deaza-6-(*p*-fluorobenzylthio)inosine	–H	–H	–H	–F	–H	–H
10. 7-Deaza-6-(*p*-chlorobenzylthio)inosine	–H	–H	–H	–Cl	–H	–H
11. 7-Deaza-6-(*p*-bromobenzylthio)inosine	–H	–H	–H	–Br	–H	–H
12. 7-Deaza-6-(*p*-methybenzylthio)inosine	–H	–H	–H	$-CH_3$	–H	–H
13. 7-Deaza-6-(*p*-butylbenzylthio)inosine	–H	–H	–H	$-{}^tBu$	–H	–H
14. 7-Deaza-6-(*p*-nitrobenzylthio)inosine	–H	–H	–H	$-NO_2$	–H	–H
15. 7-Deaza-6-(*p*-cyanobenzylthio)inosine	–H	–H	–H	–CN	–H	–H
16. 7-Deaza-6-(*p*-methoxycarbonylbenzylthio)inosine	–H	–H	–H	$-CO_2CH_3$	–H	–H
17. 7-Deaza-6-(*p*-methoxybenzylthio)inosine	–H	–H	–H	$-OCH_3$	–H	–H
18. 7-Deaza-6-(*p*-trifluoromethoxybenzylthio)inosine	–H	–H	–H	$-OCF_3$	–H	–H
19. 7-Deaza-6-(*p*-vinylbenzylthio)inosine	–H	–H	–H	$-CH{=}CH_2$	–H	–H
20. 7-Deaza-6-(2,4-chlorobenzylthio)inosine	–H	–Cl	–H	–Cl	–H	–H
21. 7-Deaza-6-(3,4-chlorobenzylthio)inosine	–H	–H	–Cl	–Cl	–H	–H
22. 7-Deaza-6-(2-fluoro-6-chlorobenzylthio)inosine	–H	–F	–H	–H	–H	–Cl
23. 7-Deaza-6-(2,4-fluorobenzylthio)inosine	–H	–F	–H	–F	–H	–H
24. 7-Iodo-7-deaza-6-benzylthioinosine	–I	–H	–H	–H	–H	–H

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% FBS.

2.4. *Evaluation of 7-deaza-6-benzylthioinosine analogues as alternative substrates for purified T. gondii adenosine kinase*

The assay mixture contained 50 mM Tris–Cl, pH 7.5, 0.25 mM ATP; 5 mM $MgCl_2$; 1 mM DTT, 25 mM NaF, 100 μM of the analogue under study, and 100 μL of purified recombinant enzyme prepared as previously described [18], in a final volume of 200 μL. Incubation was carried out overnight 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 the substrate and products in the supernatant were separated by HPLC.

2.5. *HPLC analysis of the products of the enzyme assays*

Hewlett Packard 1050 HPLC systems equipped with autosamplers, autoinjectors, quaternary pumps, multiple wave length diode array base triple channel UV monitors were 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 Hypersil (25 cm × 0.46 cm, ODS 5 μm) C_{18} reverse phase column (Jones Chromatography, Lakewood, Colorado). A 100-μL sample is injected. Elution is carried out for 35 min with a flow rate of 1 mL/min starting with a 30 min linear gradient of Buffer A (50 mM ammonium acetate, 0.5% acetonitrile, pH 3.0), to Buffer B (50 mM ammonium acetate, 60% acetonitrile, pH 4.8). This was followed by a 5-min runtime of 100% Buffer A. 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 λ_{max}/254 nm. Mass spectrometry analyses were used to verify the identity of the products as described below.

2.6. Mass spectrometry analyses

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

2.7. Evaluation of 7-deaza-6-benzylthioinosine and its analogues as potential antitoxoplasmosis agents against T. gondii in tissue culture

The wild type RH and adenosine kinase deficient mutant $TgAK^{-3}$ [17] 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 antitoxoplasmosis agents were determined by measuring 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, Rockville, MD), grown for no more than 20 passages in RPMI 1640 medium [14,15]. The viability of intracellular parasites was evaluated by the selective incorporation of radiolabeled uracil into the nucleic acids of the parasites (minimum of triplicate assays) as previously described [13–15,19]. Briefly, confluent cells (4–5 day incubation) were cultured for 24 h in the 24-well flat bottom microtiter plates ($\sim 5 \times 10^5$/(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 a 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 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, Hillsboro, OR), which were pre-washed each with 1-mL double distilled H_2O and dried. After filtration, the filters were washed with 10 mL of 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 a percent reduction in the uptake of radiolabeled uracil by treated parasites as compared to the untreated controls [13–15,19]. Radiolabel incorporation closely correlates with parasite growth [20]. The IC_{50} (the concentration causing 50% inhibition) values were estimated by a polynomial equation from dose response data from at least three separate experiments.

2.8. Host toxicity of 7-deaza-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 (minimum of triplicate assays) 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 [13–15,19]. Briefly, confluent cells were incubated for at least 24 h in 96-well flat bottom microtiterplates ($\sim 10^5$/(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).

3. Results and discussion

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

7-Deaza-6-benzylthioinosine (**1**) and its analogues (Table 1) were tested as alternative substrates of *T. gondii* adenosine kinase. HPLC analysis of the substrates and products of the enzyme assays demonstrated that 7-deaza-6-benzylthioinosine (**1**) and its analogues were converted to their respective nucleoside 5′-monophosphates by *T. gondii* adenosine kinase. Fig. 1 shows the reversed-phase HPLC profile of the metabolism of one of these analogues, 7-deaza-6-(*p*-fluorobenzylthio)inosine (**9**), to its 5′-monophosphate derivative. In the control (Panel A) 7-deaza-6-(*p*-fluorobenzylthio)inosine (**9**) eluted at 28 min and there was no traces of its 5′-monophosphate derivative. In the experimental assay (Panel B), 7-deaza-6-(*p*-fluorobenzylthio)inosine (**9**) eluted at 28 min and its 5′-monophosphate derivative appeared as a new peak at 22.5 min. As expected, 7-deaza-6-(*p*-fluorobenzylthio)inosine (**9**) and its 5′-monophosphate derivative had higher absorbance at 297 nm, the λ_{max} of 7-deaza-6-(*p*-fluorobenzylthio)inosine, than at 254 nm. Time course studies showed that there was a decrease in the amount of 7-deaza-6-(*p*-fluorobenzylthio)inosine with time accompanied by an increase in the formation of 7-deaza-6-(*p*-fluorobenzylthio)inosine 5′-monophosphate. The other analogues have similar metabolic

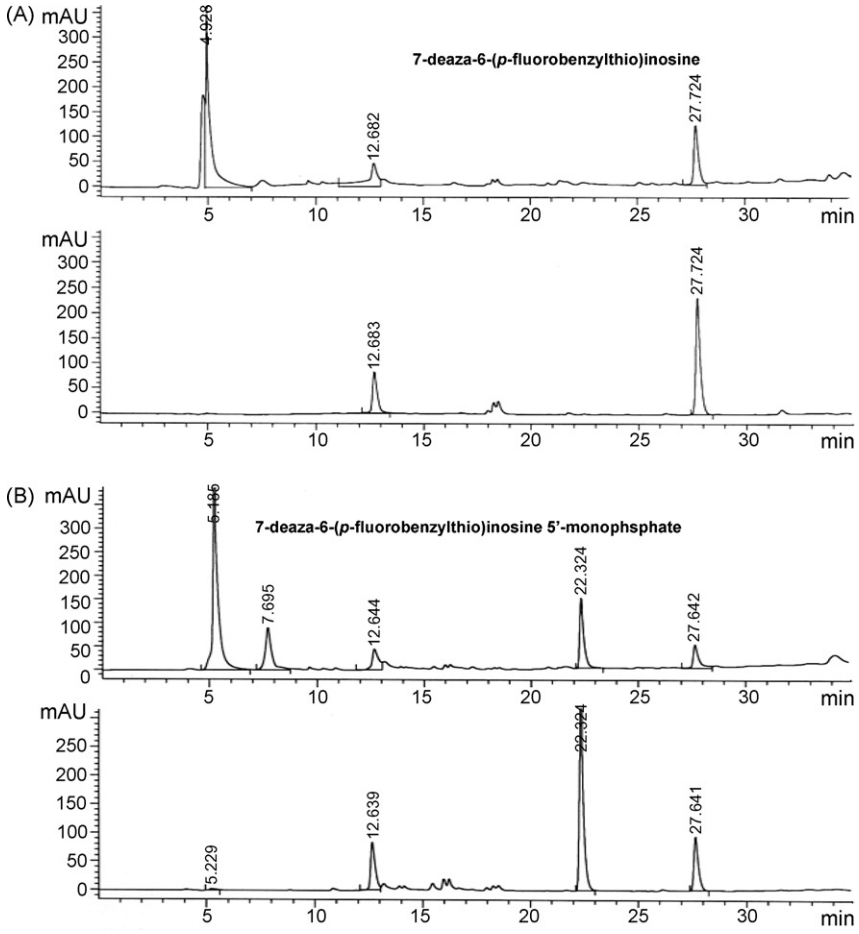


Fig. 1 – The reversed-phase HPLC profile of the metabolism of 7-deaza-6-(*p*-fluorobenzylthio)inosine to its 5′-monophosphate. (A) Controls, 7-deaza-6-(*p*-fluorobenzylthio)inosine in reaction mixture after incubation without the toxoplasma adenosine kinase. (B) Experimental, 7-deaza-6-(*p*-fluorobenzylthio)inosine 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 297 nm, the λ_{max} of 7-deaza-6-(*p*-fluorobenzylthio)inosine.

profiles although the retention time and amount of nucleoside 5′-monophosphates synthesized varied between different compounds. The chemical structures of the metabolites as the respective nucleoside 5′-monophosphates of the compound studied were verified by mass spectrometry. Fig. 2 shows the negative electrospray mass spectrum of 7-deaza-6-(*p*-fluorobenzylthio)inosine 5′-monophosphate and its (M–H) ion at 470.146. The metabolism of 7-deaza-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, apparent K_i values presented in Table 2 are equal to apparent K_m values [21].

3.2. *Evaluation of antitoxoplasmic activity and host toxicity in tissue culture*

7-Deaza-6-benzylthioinosine (**1**) and its analogues (**2–26**) were evaluated as potential antitoxoplasmic 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 efficacies, of these compounds (estimated as IC_{50}) 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 [13–15,19,20]. Therefore, an exponential increase in radiolabel incorporation closely correlates with the exponential growth of the parasite [20].

The results in Table 2 show that the antitoxoplasma efficacy (IC_{50}) of **1** (7-deaza-6-benzylthioinosine, 11.6 μM) was better than that of the parent compounds, **26** (6-benzylthioinosine, 13.3 μM) and **25** (7-deazainosine, 36.1 μM). Previous structure–activity relationship studies [12] demonstrated that the absence of N7 allowed flexibility in the movement of the bulky 6-benzylthio group to fit better in the hydrophobic pocket of *T. gondii* adenosine kinase. This finding also demonstrates that the N7 of the purine ring is not a critical structural requirement for antitoxoplasmic activity. On the other hand, 7-iodo-7-deaza-6-benzylthioinosine (**24**) exhibited

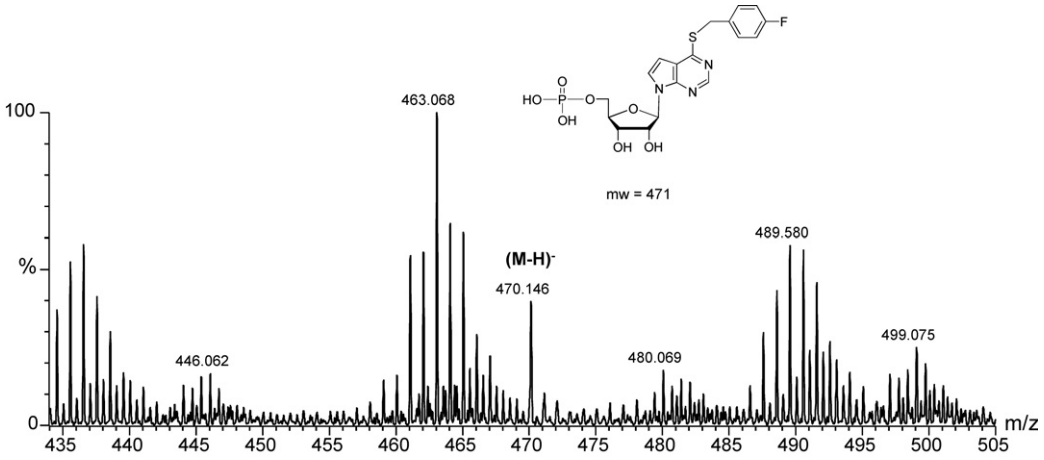


Fig. 2 – Negative electrospray mass spectrum of 7-deaza-6-(*p*-fluorobenzylthio)inosine 5′-monophosphate showing the (M–H) ion at 470.146 among salt clusters.

Table 2 – Binding affinity (apparent K_i) of 7-deaza-6-benzylthioinosine and analogues to *Toxoplasma gondii* adenosine kinase and the effect of different doses of these compounds on host toxicity and percent survival of wild type and adenosine kinase deficient (*TgAK*$^{-3}$) strains of *Toxoplasma gondii* grown in human fibroblasts in culture

Compound	K_i[a] (μM)	Infection	Concentration (μM)					IC_{50} (μM)
			0	5	10	25	50	
1. 7-Deaza-6-benzylthioinosine	28 ± 3.6	Wild type	100	82.9	51.8	0.0	0.0	11.6 ± 0.8
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
2. 7-Deaza-6-(*o*-fluorobenzylthio)inosine	59 ± 6.1	Wild type	100	99.2	93.7	37.3	15.4	20.5 ± 2.6
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	85.8	
3. 7-Deaza-6-(*o*-chlorobenzylthio)inosine	36 ± 5.7	Wild type	100	97.2	66.4	15.1	3.5	14.3 ± 0.5
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
4. 7-Deaza-6-(*o*-bromobenzylthio)inosine	106 ± 17	Wild type	100	100	90.8	67.4	47.7	49.0 ± 10.1
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	99.2	
5. 7-Deaza-6-(*o*-methylbenzylthio)inosine	228 ± 49	Wild type	100	100	100	99.2	95.2	[b]
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	96.1	
6. 7-Deaza-6-(*m*-nitrobenzylthio)inosine	45 ± 8.9	Wild type	100	100	100	82.7	42.6	45.4 ± 4.7
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	98.1	
7. 7-Deaza-6-(*m*-methylbenzylthio)inosine	8.5 ± 1.4	Wild type	100	58.0	9.5	0.0	0.0	5.7 ± 0.8
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	90.6	
8. 7-Deaza-6-(*m*-trifluoromethylbenzylthio)inosine	22 ± 4.0	Wild type	100	100	97.1	50.7	43.6	33.0 ± 3.5
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
9. 7-Deaza-6-(*p*-fluorobenzylthio)inosine	38 ± 14	Wild type	100	89.6	58.4	22.1	9.4	13.7 ± 0.4
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
10. 7-Deaza-6-(*p*-chlorobenzylthio)inosine	11 ± 1.1	Wild type	100	70.4	14.4	0.0	0.0	7.5 ± 1.6
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
11. 7-Deaza-6-(*p*-bromobenzylthio)inosine	253 ± 56	Wild type	100	100	100	100	98.8	[b]
		TgAK^{-3}	100	99.0	100	100	100	
		None	100	100	100	100	100	

Table 2 (*Continued*)

Compound	K_i[a] (μM)	Infection	Concentration (μM)					IC_{50} (μM)
			0	5	10	25	50	
12. 7-Deaza-6-(*p*-methybenzylthio)inosine	19 ± 3.9	Wild type	100	80.1	17.4	1.7	0.0	8.8 ± 0.7
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
13. 7-Deaza-6-(*p*-butylbenzylthio)inosine	24 ± 2.1	Wild type	100	87.5	29.8	6.7	0.0	10.2 ± 0.4
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
14. 7-Deaza-6-(*p*-nitrobenzylthio)inosine	35 ± 9.5	Wild type	100	85.6	56.7	25.4	10.7	13.5 ± 0.6
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	99.4	88.7	87.1	
15. 7-Deaza-6-(*p*-cyanobenzylthio)inosine	5.2 ± 1.1	Wild type	100	52.5	4.0	0.0	0.0	5.3 ± 0.6
		TgAK^{-3}	100	98.4	100	100	100	
		None	100	100	100	94.3	82.1	
16. 7-Deaza-6-(*p*-methoxycarbonylbenzylthio)inosine	7.0 ± 1.3	Wild type	100	53.7	10.2	0.0	0.0	5.0 ± 1.7
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	98.2	96.7	89.3	
17. 7-Deaza-6-(*p*-methoxybenzylthio)inosine	5.8 ± 1.0	Wild type	100	49.8	5.0	0.0	0.0	4.6 ± 0.2
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	90.6	
18. 7-Deaza-6-(*p*-trifluoromethoxybenzylthio)inosine	35 ± 14	Wild type	100	87.3	59.4	25.8	13.4	14.0 ± 2.0
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	73.1	
19. 7-Deaza-6-(*p*-vinylbenzylthio)inosine	180 ± 30	Wild type	100	100	100	94.2	88.8	[b]
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
20. 7-Deaza-6-(2,4-chlorobenzylthio)inosine	26 ± 7.6	Wild type	100	82.3	51.7	12.9	3.5	11.3 ± 0.9
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
21. 7-Deaza-6-(3,4-chlorobenzylthio)inosine	28 ± 11	Wild type	100	83.6	51.0	13.4	3.3	11.5 ± 1.4
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	90.6	93.2	
22. 7-Deaza-6-(2-fluoro-6-chlorobenzylthio)inosine	7.2 ± 1.3	Wild type	100	52.5	9.5	0.0	0.0	4.9 ± 0.9
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	94.3	
23. 7-Deaza-6-(2,4-fluorobenzylthio)inosine	16 ± 3.9	Wild type	100	76.1	17.9	0.0	0.0	8.3 ± 0.1
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
24. 7-Iodo-7-deaza-6-benzylthioinosine	83 ± 4.2	Wild type	100	100	100	100	78.3	31.5 ± 7.7
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
25. 7-Deazainosine	130 ± 24	Wild type	100	95.6	82.5	59.6	41.2	36.1 ± 3.7
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	100	100	100	
26. 6-Benzylthioinosine	38 ± 3.6	Wild type	100	98.8	54.4	28.8	1.8	13.3 ± 0.5
		TgAK^{-3}	100	100	100	100	100	
		None	100	100	99.7	99.4	98.8	
Sulfadiazine		Wild type	100	92.9	58.0	53.4	46.3	27.3 ± 3.3
		None	100	98.2	99.8	99.8	102	
Pyrimethamine		Wild type	100	98.9	55.3	25.1	23.2	16.1 ± 2.5
		None	100	101	100	108	108	

Host toxicity of uninfected cells was measured by MTT method in at least two independent experiments each of three replica as previously described [13–15,19].

Percent survival of parasites was measured by incorporation of [5,6-^{13}H]uracil in at least two independent experiments of three replica each as previously described [13–15,19].

[a] Data from Ref. [12].

[b] Could not be determined from available data.

a lesser efficacy (31.5 μM) than 7-deaza-6-benzylthioinosine (**1**, 11.6 μM). This was unexpected since the 7-iodo-7-deaza derivative of adenosine (7-iodotubercidin) is a better substrate than 7-deazaadenosine (tubercidin) for *T. gondii* adenosine kinase [10]. The decrease in antitoxoplasma efficacy of 7-iodo-7-deaza-6-benzylthioinosine (**24**) could be due to the presence of both the 7-iodo and 6-benzyl groups which decreased ligand-fit of this compound as a substrate for *T. gondii* adenosine kinase [12].

The results in Table 2 also show that all the 7-deaza-6-benzylthioinosines, except **5** (*o*-methyl), **11** (*p*-bromo) and **19** (*p*-vinyl), were effective against infection with the wild type (RH) *T. gondii* and the inhibition was dose-dependent. The most potent analogues were **17** (7-deaza-6-(*p*-methoxybenzylthio)inosine, 4.6 μM) **16** (7-deaza-6-(*p*-methoxycarbonylbenzylthio)inosine, 5.0 μM) and **15** (7-deaza-6-(*p*-cyanobenzylthio)inosine, 5.3 μM). Moreover, none of the active compounds was effective against infection with adenosine kinase deficient strain ($TgAK^{-3}$). The lack of sensitivity of $TgAK^{-3}$ to these purine nucleoside analogues is not due to 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 [14]. Therefore, it can be concluded that the presence of *T. gondii* adenosine kinase is a requirement for the 7-deaza-6-benzylthioinosines to exert their antitoxoplasmic effect. Consequently, these compounds are substrates for *T. gondii* adenosine kinase *in vivo* as was the case with the *in vitro* enzyme assays [12]. These results confirm further the HPLC analyses which demonstrated that these 7-deaza-6-benzylthioinosines are converted to their respective 5′-monophosphate by *T. gondii* adenosine kinase, which is a prerequisite for these analogues to exert their antitoxoplasmic effect.

In general, the degree of antitoxoplasmic efficacies of the 7-deaza-6-benzylthioinosines (Table 2) correlated well with their K_i values which, in turn, were influenced by the nature and position of substituents on the 6-benzyl group. Single substitutions at the *ortho* position (**2–5**) led to the loss of antitoxoplasmic efficacy (IC_{50}) when compared to the unsubstituted compound **1**. The weaker ligands in this series were **5** (*o*-methyl, no effect) and **4** (*o*-bromo, 49 μM). Notably, when the methyl group at the *ortho* position (not effective, **5**) was moved to the *meta* (**7**, 5.7 μM) or the *para* (**12**, 8.8 μM) position, a substantial increase in efficacy was observed. At the *meta* position, an electron-donating substituent (**7**, 5.7 μM) resulted in a better binding affinity than an electron-withdrawing substituent (**6**, 45 μM and **8**, 33 μM). At the *para* position, a Cl substitution (**10**, 7.5 μM) exhibited somewhat increased binding affinity in comparison to a F substitution (**9**, 13.7 μM). Interestingly, the Br substitution (**11**) resulted in almost complete loss of antitoxoplasmic efficacy. A similar trend was also observed for halogen substitutions in the *ortho*-substituted compounds **2–4**, and the order of the potency was Cl > F ≫ Br. In addition to electronic effects, it appeared that the relative size of the halogen substituents might have also have a role in how they affect the antitoxoplasmic efficacy of the 7-deaza-6-benzylthioinosines. The best result was achieved when a methoxy group (**17**, 4.6 μM), a methoxycarbonyl group (**16**, 5.0 μM) or a cyano group (**15**, 5.3 μM) was added to the *para* position. In the case of di-substituted analogues **20–23**, di-*ortho* substitutions (**22**, 4.9 μM) showed increased binding affinity in comparison to the *ortho–para* di-substitutions (**23**, 8.3 μM and **20**, 11.3 μM) or the *meta–para* di-substitutions (**21**, 11.5 μM). These results indicate that the incorporation of the 7-deazapurine motif led to improvement in the binding affinity of the 6-benzylthioinosines. Furthermore, the functionality of the 6-benzylthio group on the 7-deazapurine moiety was extended by the addition of substituents such as *p*-cyano (**15**) and *p*-methoxy (**17**). Previous studies [11,12,19] demonstrated that such substituents provide additional proper interactions with the surrounding residues in the binding site of *T. gondii* adenosine kinase, and are conducive to enhancing the binding affinity of the 7-deaza-6-benzylthioinosines. Finally, the results in Table 2 show that several compounds (e.g., **15**, **16** and **17**) were more active than sulfadiazine.

Table 2 also shows that none of the compounds had 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 active substrates for mammalian adenosine kinase [11,13,14]. 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. Indeed, our previous studies have demonstrated that NBMPR is transported and metabolized only by cells infected with wild type toxoplasma [15]. On the other hand, NBMPR is neither transported nor metabolized by uninfected host cells or cells infected with mutant parasites lacking the adenosine/purine transporter [15]. It was concluded that 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 [15]. Therefore, the newly synthesized compounds may act similarly to NBMPR and not gain entry to uninfected host cells to exert toxic side-effects.

It is clear from the present study that analogues of 7-deaza-6-benzylthioinosine have antitoxoplasmic effect and they work by a different mechanism than the other known antitoxoplasmic drugs. The mechanism and/or targets of toxicities of 7-deaza-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. The nucleotides of 7-deaza-6-benzylthioinosine and its analogues could interfere with, among other things, other steps of 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 being conducted in our laboratories to address these questions.

In summary, the present results demonstrate that the incorporation of the 7-deaza system led to improved antitoxoplasmic efficacy of 6-benzylthioinosine. The 7-deaza-6-benzylthioinosine and its analogues exhibited selective antitoxoplasmic effects in cell culture. The efficacy of these compounds as selective antitoxoplasmic agents varied according to the nature and position of various substituents on the phenyl ring of the benzyl group. The *ortho*-substitutions appeared to be less important for antitoxoplasmic activity,

while the *meta*-substitutions exhibited improved cellular activity as compared to the parent compound 7-deaza-6-benzylthioinosine (**1**). The observations made in these studies can provide important guidance to further development of antitoxoplasma agents.

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