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Synthesis, biological evaluation and molecular modeling studies of *N*⁶-benzyladenosine analogues as potential anti-toxoplasma agents

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Abstract

Toxoplasma gondii is an opportunistic pathogen responsible for toxoplasmosis. *T. gondii* is a purine auxotroph incapable of *de novo* purine biosynthesis and depends on salvage pathways for its purine requirements. Adenosine kinase (EC.2.7.1.20) is the major enzyme in the salvage of purines in these parasites. 6-Benzylthioinosine and analogues were established as “subversive substrates” for the *T. gondii*, but not for the human adenosine kinase. Therefore, these compounds act as selective anti-toxoplasma agents. In the present study, a series of *N*⁶-benzyladenosine analogues were synthesized from 6-chloropurine riboside with substituted benzylamines & – solution phase parallel synthesis. These *N*⁶-benzyladenosine analogues were evaluated for their binding affinity to purified *T. gondii* adenosine kinase. Furthermore, the anti-toxoplasma efficacy and host toxicity of these compounds were tested in cell culture. Certain substituents on the aromatic ring improved binding affinity to *T. gondii* adenosine kinase when compared to the unsubstituted *N*⁶-benzyladenosine. Similarly, varying the type and position of the substituents on the aromatic ring led to different degrees of potency and selectivity as anti-toxoplasma agents. Among the synthesized analogues, *N*⁶-(2,4-dimethoxybenzyl) adenosine exhibited the most favorable anti-toxoplasma activity without host toxicity. The binding mode of the synthesized *N*⁶-benzyladenosine analogues were characterized to illustrate the role of additional hydrophobic effect and van der Waals interaction within an active site of *T. gondii* adenosine kinase by induced fit molecular modeling.

Keywords

Toxoplasma gondii adenosine kinase; Toxoplasmosis; Chemotherapy; *N*⁶-Benzyladenosine analogues; Molecular modeling

1. Introduction

Toxoplasma gondii is an opportunistic pathogen responsible for toxoplasmosis. Toxoplasmosis is known to be one of the most prevalent parasitic infections of the central nervous system and causes severe congenital defects in unborn children of infected mothers as well as lethal

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encephalitis in immunocompromised patients such as acquired immunodeficiency syndrome (AIDS) [1–3]. Pyrimethamine is currently used in combination with sulfa drugs such as sulfadiazine in toxoplasmic infections [4,5]. This combination therapy targets enzymes of folate metabolism such as dihydrofolate reductase [6–9]. However, current therapies have been limited due to resistance, host toxicity and side effects. Particularly, sulfa drugs cause severe side effects such as skin allergy and bone marrow suppression [10,11]. To overcome such disadvantages, it is desirable to develop novel drug candidates based on different mechanism of action with high degree of selectivity.

T. gondii adenosine kinase is an attractive target for the treatment of toxoplasmosis [12,13] for the following reasons. First, *T. gondii* is a purine auxotroph and depends on the salvage pathways for its purine requirements, and the salvage of adenosine is the major source of purines in *T. gondii* [14,15]. Specifically, the activity of adenosine kinase (EC.2.7.1.20), the enzyme responsible for the salvage of adenosine in this parasite, is 10-fold higher than those of other enzymes in the purine salvage pathways, and hence contributes more significantly to the salvage of purines in *T. gondii* than any other enzyme of the purine salvage pathways [12–16]. Secondly, structure-activity relationships [17,18], comparative enzymatic [18–20], metabolic [19–21], molecular [22], and X-ray structural studies [23–25] have demonstrated that there are sufficient differences between the active sites and substrate specificities of human and *T. gondii* adenosine kinases that can be exploited to design specific “subversive substrates” for the *T. gondii* enzyme [12,13].

T. gondii adenosine kinase is a 363-residue (39.3 kDa) monomeric protein [22], that catalyzes the phosphorylation of adenosine to adenosine 5'-monophosphate (AMP), using the γ -phosphate group of ATP as the phosphate donor [12,13]. The overall enzyme structure consists of two domains: a large domain and a small (lid) domain as shown in Fig. 1a. The catalytic site for adenosine and ATP is located at the domain interfaces. The overlay structures of apo enzyme (apo enzyme: 1LIO) and the enzyme:adenosine:ATP complex (complex: 1LII) suggest that there is a movement of the small domain towards the large domain (Fig. 1a), which forms the closed conformation causing translocation of Arg136 towards the γ -phosphate of ATP to initiate catalysis (Fig. 1b). Consequently, ATP acquires extended conformation and orientation toward the 5'-OH of adenosine for its phosphorylation as shown in Fig. 1b. The adenosine binding pocket is composed of the ribose binding hydrophilic region (Asp24, Gly69, and Asn73), the purine binding region [23], and an additional hydrophobic region [18]. The purine motif of adenosine is surrounded by some of the hydrophobic residues such as Tyr169 and Ile22, that forms aromatic stacking by π - π and C-H- π interaction (Fig. 2a).

Our previous structure-activity relationships and molecular modeling studies of a series of 6-benzylthioinosine analogues with *T. gondii* adenosine kinase [17,18] emphasized the importance of the hydrophobic effect of the benzyl moiety at the 6-position of the purine ring as well as the type and position of substituents on the benzyl moiety in enhancing the binding affinity of 6-benzylthioinosine analogues to *T. gondii* adenosine kinase. These studies also furthered our understanding of the structural requirements for the binding of ligands by induced fit mechanism.

In the present study, we report the synthesis of *N*⁶-benzyladenosine analogues by solution phase parallel synthesis with the goal of identifying anti-toxoplasma agents. We have also carried out systematic characterization of the hydrophobic effect and van der Waals interaction employing an induced fit molecular modeling approach to demonstrate the role of additional interaction in enhancing the binding affinity of ligands to *T. gondii* adenosine kinase in this series.

2. Materials and methods

2.1. Reagents

[8-¹⁴C]Adenosine (55 Ci/mol) and [5,6-¹³H]uracil were purchased from Moravak Biochemicals. RPMI-1640 medium from GIBCO BRL; fetal bovine serum (FBS) from HyClone Laboratories. All other chemicals and compounds were obtained from Sigma Chemical Co. or Fisher Scientific.

2.2. Chemical synthesis

2.2.1. General—Solution phase synthesis was performed on Argonaut Quest 210 Organic Synthesizer. Melting points were determined on a Mel-temp II apparatus and are uncorrected. Optical rotation was determined on a Jasco DIP-370 Digital Polarimeter. UV spectra were obtained on a Beckman DU-650 spectrophotometer. NMR spectra were recorded on a Varian Inova 500 spectrometer and chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane as internal reference. Chemical shifts (δ) are reported as s (singlet), d (doublet), t (triplet), m (multiplet), or bs (broad singlet). TLC was performed on Uniplates (silica gel) purchased from Analtech Co. Silica gel 60 (220–440 mesh) was used for flash column chromatography. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA.

2.2.2. *N,N*-Di-*tert*-butyl[(2-fluoro-4-nitro)benzylamino]dicarboxylate (2a)—A solution of 2-fluoro-4-nitrotoluene (300 mg, 1.93 mmol) in CCl₄ (4.8 mL) was treated with *N*-bromosuccinimide (413 mg, 2.32 mmol) and benzoyl peroxide (46.8 mg, 0.193 mmol). The mixture was heated to reflux for 8 h. The insoluble material was filtered and the filtrate was washed with brine. The organic layer was dried over MgSO₄ and then concentrated. The crude product was dissolved in THF (7.0 mL) and was cooled to 0°C. To this solution, di-*tert*-butyl iminodicarboxylate (462 mg, 2.13 mmol) and sodium hydride (51.0 mg, 2.13 mmol) in THF (21.0 mL) were added. The reaction mixture was stirred at 0°C for 1 h followed by stirring at room temperature for 7 h. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with brine and then dried over MgSO₄. After concentration, the residue was purified by silica gel column chromatography (*n*-Hexane:EtOAc = 95:5) to give **2a** (358 mg, 50% for 2 steps): ¹H NMR (500 MHz, CDCl₃) δ 1.48 (s, 18H), 4.93 (s, 2H), 7.42 (t, *J* = 7.8 Hz, 1H), 7.92 (dd, *J*₁ = 10 Hz, *J*₂ = 2.5 Hz, 1H), 8.02 (dd, *J*₁ = 8.5 Hz, *J*₂ = 2.5 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 27.9, 43.2, 83.3, 111.1 (d, *J* = 27.1 Hz), 119.3, 128.7, 133.5, 147.7 (d, *J* = 8.6 Hz), 152.1, 159.6 (d, *J* = 249.4 Hz).

2.2.3. *N,N*-Di-*tert*-butyl[(2-chloro-4-cyano)benzylamino]dicarboxylate (2b)—Compound **2b** was prepared under the same procedure outlined above for compound **2a** (52% for 2 steps): ¹H NMR (500 MHz, CD₃OD) δ 1.48 (s, 18H), 4.92 (s, 2H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.75 (d, *J* = 8.5 Hz, 1H), 7.89 (s, 1H); ¹³C NMR (125 MHz, CD₃OD) δ 26.8, 47.0, 83.2, 112.0, 116.9, 127.4, 130.7, 132.5, 132.9, 141.8, 152.1.

2.2.4. (2-Fluoro-4-nitro)benzylamine hydrochloride (3a)—A solution of compound **2a** (800 mg, 2.16 mmol) in MeOH (21.6 mL) was treated with 1 N HCl/Et₂O (21.6 mL). The reaction mixture was stirred for 5 h at room temperature and then concentrated. The residue was triturated with ether and then filtered to give **3a** (379 mg, 85%): UV (MeOH) λ_{max} 255.0 nm; ¹H NMR (500 MHz, DMSO-*d*₆) δ 4.21 (s, 2H), 7.91 (t, *J* = 7.8 Hz, 1H), 8.23 (m, 2H), 8.64 (bs, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 35.9, 111.7 (d, *J* = 26.6 Hz), 120.1, 129.4, 132.7, 148.9 (d, *J* = 9.1 Hz), 160.1 (d, *J* = 249.9 Hz).

2.2.5. (2-Chloro-4-cyano)benzylamine hydrochloride (3b)—Compound **3b** was prepared under the same procedure outlined above for compound **3a** (83%): UV (MeOH)

$\lambda_{\max}$ 284.0 nm; ^1H NMR (500 MHz, DMSO- d_6) δ 4.22 (s, 2H), 7.86 (d, J = 8.0 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 8.19 (s, 1H), 8.81 (bs, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 40.2, 113.2, 117.7, 131.5, 131.7, 133.2, 134.1, 137.9.

2.2.6. (2-Fluoro-4-methoxy)benzylalcohol (5)—A solution of (2-fluoro-4-methoxy)benzaldehyde (4.50 g, 29.2 mmol) in MeOH (73.0 mL) was treated with NaBH_4 (1.66 g, 43.8 mmol) at 0°C . After 30 min, the reaction mixture was stirred for 3 h at room temperature. The reaction mixture was quenched with water and then concentrated. The residue was diluted with EtOAc and then washed with 5% HCl and brine. The organic layer was dried over MgSO_4 and concentrated. The residue was purified by silica gel column chromatography (*n*-Hexane:EtOAc = 7:1 $\rightarrow$ 4:1) to give **5** (4.38 g, 96%): ^1H NMR (500 MHz, CDCl_3) δ 2.50 (bs, 1H), 3.78 (s, 3H), 4.62 (s, 2H), 6.60 (dd, J_1 = 11.5 Hz, J_2 = 2.5 Hz, 1H), 6.66 (dd, J_1 = 8.0 Hz, J_2 = 2.5 Hz, 1H), 7.26 (t, J = 8.8 Hz, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 55.5, 58.8, 101.6 (d, J = 25.3 Hz), 109.7, 119.9 (d, J = 15.3 Hz), 130.3, 160.5, 161.3 (d, J = 245.1 Hz).

2.2.7. *N,N*-Di-*tert*-butyl[(2-fluoro-4-methoxy)benzylamino]dicarboxylate (6)—A solution of (2-fluoro-4-methoxy)benzylalcohol **5** (3.21 g, 20.6 mmol) in CH_2Cl_2 (69.0 mL) was treated with carbon tetrabromide (8.18 g, 24.7 mmol) and triphenylphosphine (6.47 g, 24.7 mmol). The reaction mixture was stirred for 3 h at room temperature. The insoluble material was filtered and the filtrate was washed with brine. The organic layer was dried over MgSO_4 and then concentrated. The crude product was dissolved in THF (76.0 mL) and was cooled to 0°C . To this solution, di-*tert*-butyl iminodicarboxylate (4.91 g, 22.6 mmol) and sodium hydride (0.543 g, 22.6 mmol) in THF (228.0 mL) were added. The reaction mixture was stirred at 0°C for 1 h followed by stirring at room temperature for 7 h. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with brine and then dried over MgSO_4 . After concentration, the residue was purified by silica gel column chromatography (*n*-Hexane:EtOAc = 95:5) to give **6** (4.02 g, 55% for 2 steps): ^1H NMR (500 MHz, CDCl_3) δ 1.49 (s, 18H), 3.81 (s, 3H), 4.81 (s, 2H), 6.61 (m, 1H), 6.68 (m, 1H), 7.20 (t, J = 8.5 Hz, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 27.9, 42.8, 55.5, 82.5, 101.3 (d, J = 25.3 Hz), 109.7, 117.4 (d, J = 14.8 Hz), 129.3, 152.4, 160.0, 160.9 (d, J = 252.8 Hz).

2.2.8. (2-Fluoro-4-methoxy)benzylamine hydrochloride (7)—Compound **7** was prepared under the same procedure outlined above for compound **3a** (86%): UV (MeOH) $\lambda_{\max}$ 270.0 nm; ^1H NMR (500 MHz, DMSO- d_6) δ 3.81 (s, 3H), 4.00 (s, 2H), 6.88 (dd, J_1 = 9.0 Hz, J_2 = 2.5 Hz, 1H), 6.94 (dd, J_1 = 12.0 Hz, J_2 = 2.5 Hz, 1H), 7.50 (t, J = 9.0 Hz, 1H), 8.33 (bs, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 35.7, 56.3, 102.0 (d, J = 24.9 Hz), 111.0, 113.1 (d, J = 15.3 Hz), 132.6, 161.5, 161.6 (d, J = 244.8 Hz).

2.2.9. 4-Vinylbenzylamine hydrochloride (9)—A solution of 4-vinylbenzyl chloride (1.50 g, 9.83 mmol) in *N,N*-dimethylformamide (25.0 mL) was treated with sodium azide (1.28 g, 19.7 mmol) and was stirred for 1 h at room temperature. The reaction mixture was diluted with water and then extracted with EtOAc. The organic layer was washed with brine, dried over MgSO_4 and then concentrated. The crude product was dissolved in benzene (10.0 mL) and then triphenylphosphine (3.87 g, 14.7 mmol) and water was added. The reaction mixture was heated at 70°C for 3 h and then extracted with 2 N HCl. The aqueous layer was washed with CH_2Cl_2 , basified with NaOH, and extracted with CH_2Cl_2 . The combined organic layers were concentrated, the residue was dissolved in Et_2O and 3 N HCl was added dropwise. The resulting precipitate was filtered and dried to give **9** (1.03 g, 62% for 2 steps): UV (MeOH) $\lambda_{\max}$ 248.0 nm; ^1H NMR (500 MHz, DMSO- d_6) δ 4.02 (s, 2H), 5.32 (d, J = 11.0 Hz, 1H), 5.90 (d, J = 17.5 Hz, 1H), 6.77 (dd, J_1 = 17.5 Hz, J_2 = 11.5 Hz, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.54 (d, J = 8.5 Hz, 2H), 8.42 (bs, 3H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 42.3, 115.5, 126.7, 129.7, 134.2, 136.6, 137.7.

2.2.10. General procedure for the synthesis of *N*⁶-benzyladenosine analogues (11a-v)—Compound **10** (100 mg, 0.349 mmol) was added to each reaction vessel of Argonaut Quest 210 Organic Synthesizer and then *n*-PrOH (10.0 mL) was added. To this solution, the appropriate amine (or amine hydrochloride) and triethylamine (or *N,N*-diisopropylethylamine) (0.2 mL) were added. The reaction mixtures were stirred (upward stroke = 50%, time = 4 s) at 100°C (or 80°C) for 11 h and then allowed to cool. The reaction mixtures were concentrated and the residues were purified by silica gel column chromatography (CH₂Cl₂:MeOH = 95:5) to give the desired products as white solid.

2.2.11. *N*⁶-Benzyladenosine (11a)—Yield 79%; mp 168–169°C; [α]_D²³ – 57.17 (*c* 0.22, MeOH); UV (MeOH) λ_{max} 269.0 nm; ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.57 (m, 1H), 3.69 (m, 1H), 3.98 (m, 1H), 4.17 (m, 1H), 4.64 (m, 1H), 4.73 (br, 2H), 5.21 (d, *J* = 4.5 Hz, 1H), 5.41 (m, 1H), 5.47 (d, *J* = 6.5 Hz, 1H), 5.91 (d, *J* = 6.5 Hz, 1H), 7.23 (m, 1H), 7.33 (m, 4H), 8.22 (s, 1H), 8.40 (s, 1H), 8.50 (bs, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 43.3, 62.1, 71.1, 73.9, 86.3, 88.4, 120.2, 127.1, 127.5, 128.7, 140.4, 148.9, 152.8, 154.9. Anal. Calcd for C₁₇H₁₉N₅O₄ requires C, 57.14; H, 5.36; N, 19.60; Found C, 57.28; H, 5.34; N, 19.66.

2.2.12. *N*⁶-(2-Fluorobenzyl)adenosine (11b)—Yield 73%; mp 191–192°C; [α]_D²⁵ – 64.07 (*c* 0.18, MeOH); UV (H₂O) λ_{max} 267.0 nm (ϵ 21376, pH 2), 267.0 nm (ϵ 21341, pH 7), 267.0 nm (ϵ 21615, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.18 (m, 1H), 4.65 (m, 1H), 4.79 (m, 2H), 5.23 (m, 1H), 5.40 (m, 1H), 5.49 (d, *J* = 5.0 Hz, 1H), 5.93 (d, *J* = 5.0 Hz, 1H), 7.14 (m, 1H), 7.20 (m, 1H), 7.32 (m, 2H), 8.24 (s, 1H), 8.44 (s, 1H), 8.48 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 37.3, 62.1, 71.1, 74.0, 86.4, 88.4, 115.5 (d, *J* = 21.0 Hz), 120.3, 124.7, 127.1, 129.0 (d, *J* = 8.1 Hz), 129.4, 140.5, 149.1, 152.8, 155.0, 160.5 (d, *J* = 242.8 Hz). Anal. Calcd for C₁₇H₁₈FN₅O₄ requires C, 54.40; H, 4.83; N, 18.66; Found C, 54.27; H, 4.84; N, 18.50.

2.2.13. *N*⁶-(2-Chlorobenzyl)adenosine (11c)—Yield 75%; mp 190–191°C; [α]_D²³ – 75.56 (*c* 0.12, MeOH); UV (H₂O) λ_{max} 267.0 nm (ϵ 17633, pH 2), 267.0 nm (ϵ 17792, pH 7), 267.0 nm (ϵ 18115, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.19 (m, 1H), 4.67 (m, 1H), 4.79 (m, 2H), 5.24 (m, 1H), 5.40 (m, 1H), 5.50 (d, *J* = 6.0 Hz, 1H), 5.94 (d, *J* = 4.0 Hz, 1H), 7.29 (m, 3H), 7.48 (m, 1H), 8.23 (s, 1H), 8.46 (s, 1H), 8.52 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 41.5, 62.2, 71.1, 74.0, 86.4, 88.4, 120.4, 127.6, 128.5, 128.8, 129.6, 132.2, 137.2, 140.6, 149.2, 152.8, 155.0. Anal. Calcd for C₁₇H₁₈ClN₅O₄ requires C, 52.11; H, 4.63; N, 17.87; Found C, 52.00; H, 4.65; N, 17.76.

2.2.14. *N*⁶-(2-Nitrobenzyl)adenosine (11d)—Yield 66%; mp 202–204°C; [α]_D²⁵ – 62.98 (*c* 0.04, MeOH); UV (H₂O) λ_{max} 266.0 nm (ϵ 16034, pH 2), 266.0 nm (ϵ 10095, pH 7), 266.0 nm (ϵ 9614, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.70 (m, 1H), 3.99 (m, 1H), 4.18 (m, 1H), 4.65 (m, 1H), 5.02 (m, 2H), 5.23 (m, 1H), 5.37 (m, 1H), 5.49 (d, *J* = 6.0 Hz, 1H), 5.93 (d, *J* = 5.5 Hz, 1H), 7.54 (m, 2H), 7.70 (t, *J* = 7.5 Hz, 1H), 8.08 (d, *J* = 8.0 Hz, 1H), 8.20 (s, 1H), 8.47 (s, 1H), 8.58 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 40.8, 62.1, 71.1, 74.0, 86.4, 88.4, 120.4, 125.0, 128.5, 129.4, 134.3, 135.3, 140.7, 148.6, 149.2, 152.8, 154.8. Anal. Calcd for C₁₇H₁₈N₆O₆ requires C, 50.75; H, 4.51; N, 20.89; Found C, 50.62; H, 4.60; N, 20.60.

2.2.15. *N*⁶-(2-Methoxybenzyl)adenosine (11e)—Yield 76%; mp 155–157°C; [α]_D²⁵ – 64.43 (*c* 0.15, MeOH); UV (H₂O) λ_{max} 267.0 nm (ϵ 21173, pH 2), 270.0 nm (ϵ 21878, pH 7), 270.0 nm (ϵ 22118, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 3.87 (s, 3H), 4.00 (m, 1H), 4.18 (m, 1H), 4.67 (m, 1H), 4.70 (m, 2H), 5.23 (m, 1H), 5.43 (m, 1H), 5.49 (d, *J* = 6.0 Hz, 1H), 5.93 (d, *J* = 6.0 Hz, 1H), 6.87 (m, 1H), 7.01 (d, *J* = 8.5 Hz, 1H), 7.12 (m, 1H), 7.23 (m, 1H), 8.21 (s, 1H), 8.26 (s, 1H), 8.43 (s, 1H); ¹³C NMR (125 MHz,

DMSO-*d*₆) δ 38.8, 55.8, 62.2, 71.2, 74.0, 86.4, 88.5, 110.9, 120.4, 120.6, 127.2, 127.7, 128.2, 140.4, 149.0, 152.9, 155.3, 157.1. Anal. Calcd for C₁₈H₂₁N₅O₅ requires C, 55.81; H, 5.46; N, 18.08; Found C, 55.69; H, 5.31; N, 17.87.

2.2.16. N⁶-(3-Fluorobenzyl)adenosine (11f)—Yield 74%; mp 155–156°C; $[\alpha]_D^{25}$ –56.31 (*c* 0.20, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 22491, pH 2), 268.0 nm (ϵ 22885, pH 7), 268.0 nm (ϵ 23107, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.19 (m, 1H), 4.65 (m, 1H), 4.75 (m, 2H), 5.23 (m, 1H), 5.41 (m, 1H), 5.49 (m, 1H), 5.93 (d, *J* = 3.5 Hz, 1H), 7.07 (t, *J* = 8.5 Hz, 1H), 7.18 (m, 2H), 7.37 (m, 1H), 8.25 (s, 1H), 8.44 (s, 1H), 8.55 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 43.0, 62.1, 71.1, 74.0, 86.4, 88.5, 113.9 (d, *J* = 21.0 Hz), 114.2 (d, *J* = 21.5 Hz), 120.3, 123.6, 130.6 (d, *J* = 8.1 Hz), 140.5, 143.7, 149.1, 152.8, 155.0, 162.7 (d, *J* = 241.9 Hz). Anal. Calcd for C₁₇H₁₈FN₅O₄ requires C, 54.40; H, 4.83; N, 18.66; Found C, 54.30; H, 4.78; N, 18.49.

2.2.17. N⁶-(3-Methylbenzyl)adenosine (11g)—Yield 75%; mp 151–152°C; $[\alpha]_D^{26}$ –56.84 (*c* 0.23, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 17760, pH 2), 268.0 nm (ϵ 17780, pH 7), 268.0 nm (ϵ 18077, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.29 (s, 3H), 3.58 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.19 (m, 1H), 4.66 (m, 1H), 4.71 (m, 2H), 5.23 (m, 1H), 5.44 (m, 1H), 5.49 (d, *J* = 6.5 Hz, 1H), 5.93 (d, *J* = 6.0 Hz, 1H), 7.05 (d, *J* = 7.0 Hz, 1H), 7.18 (m, 3H), 8.24 (s, 1H), 8.41 (s, 1H), 8.47 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 21.5, 43.3, 62.2, 71.2, 74.0, 86.4, 88.5, 120.3, 124.7, 127.8, 128.2, 128.6, 137.7, 140.4, 149.0, 152.9, 155.0. Anal. Calcd for C₁₈H₂₁N₅O₄ requires C, 58.21; H, 5.70; N, 18.86; Found C, 58.18; H, 5.86; N, 18.75.

2.2.18. N⁶-(4-Fluorobenzyl)adenosine (11h)—Yield 77%; mp 179–181°C; $[\alpha]_D^{26}$ –62.21 (*c* 0.20, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 20123, pH 2), 268.0 nm (ϵ 20181, pH 7), 268.0 nm (ϵ 20532, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.18 (m, 1H), 4.65 (m, 1H), 4.71 (m, 2H), 5.23 (m, 1H), 5.42 (m, 1H), 5.49 (d, *J* = 5.0 Hz, 1H), 5.93 (d, *J* = 5.5 Hz, 1H), 7.15 (m, 2H), 7.40 (m, 2H), 8.25 (s, 1H), 8.42 (s, 1H), 8.52 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 42.7, 62.2, 71.1, 74.0, 86.4, 88.4, 115.4 (d, *J* = 21.0 Hz), 120.3, 129.6, 136.7, 140.4, 149.0, 152.8, 154.9, 161.6 (d, *J* = 240.9 Hz). Anal. Calcd for C₁₇H₁₈FN₅O₄ requires C, 54.40; H, 4.83; N, 18.66; Found C, 54.15; H, 4.84; N, 18.47.

2.2.19. N⁶-(4-Chlorobenzyl)adenosine (11i)—Yield 76%; mp 178–180°C; $[\alpha]_D^{25}$ –63.69 (*c* 0.16, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 21391, pH 2), 268.0 nm (ϵ 21487, pH 7), 268.0 nm (ϵ 21890, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.19 (m, 1H), 4.65 (m, 1H), 4.72 (m, 2H), 5.23 (m, 1H), 5.41 (m, 1H), 5.49 (d, *J* = 6.0 Hz, 1H), 5.93 (d, *J* = 6.5 Hz, 1H), 7.38 (m, 4H), 8.24 (s, 1H), 8.43 (s, 1H), 8.54 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 42.8, 62.2, 71.1, 74.0, 86.4, 88.4, 120.3, 128.7, 129.5, 131.7, 139.6, 140.5, 149.0, 152.8, 154.9. Anal. Calcd for C₁₇H₁₈ClN₅O₄ requires C, 52.11; H, 4.63; N, 17.87; Found C, 52.10; H, 4.65; N, 17.72.

2.2.20. N⁶-(4-Cyanobenzyl)adenosine (11j)—Yield 77%; mp 118–120°C; $[\alpha]_D^{26}$ –64.31 (*c* 0.14, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 24306, pH 2), 268.0 nm (ϵ 24776, pH 7), 268.0 nm (ϵ 25086, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.57 (m, 1H), 3.68 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.64 (m, 1H), 4.80 (m, 2H), 5.23 (m, 1H), 5.38 (m, 1H), 5.48 (d, *J* = 6.0 Hz, 1H), 5.92 (d, *J* = 6.0 Hz, 1H), 7.53 (d, *J* = 7.5 Hz, 2H), 7.79 (d, *J* = 8.5 Hz, 2H), 8.22 (s, 1H), 8.44 (s, 1H), 8.62 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 43.3, 62.1, 71.1, 74.0, 86.4, 88.4, 109.9, 119.4, 120.3, 128.4, 132.7, 140.6, 146.5, 149.1, 152.8, 154.9. Anal. Calcd for C₁₈H₁₈N₆O₄ requires C, 56.54; H, 4.74; N, 21.98; Found C, 56.52; H, 4.72; N, 21.89.

2.2.21. N⁶-(4-Trifluoromethylbenzyl)adenosine (11k)—Yield 75%; mp 151–153°C; $[\alpha]_D^{25}$ –52.82 (*c* 0.18, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 22451, pH 2), 268.0 nm (ϵ 22387, pH 7), 268.0 nm (ϵ 22659, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.58 (m, 1H), 3.70 (m, 1H), 4.00 (m, 1H), 4.18 (m, 1H), 4.65 (m, 1H), 4.81 (m, 2H), 5.23 (m, 1H), 5.39 (m, 1H), 5.49 (d, *J* = 6.0 Hz, 1H), 5.93 (d, *J* = 6.5 Hz, 1H), 7.56 (d, *J* = 7.5 Hz, 2H), 7.69 (d, *J* = 7.5 Hz, 2H), 8.23 (s, 1H), 8.44 (s, 1H), 8.62 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 43.2, 62.1, 71.1, 74.0, 86.4, 88.4, 120.3, 121.6, 123.8, 125.6, 126.0, 127.8 (q, *J* = 30.9 Hz), 128.2, 140.6, 145.4, 149.1, 152.8, 154.9. Anal. Calcd for C₁₈H₁₈F₃N₅O₄ requires C, 50.83; H, 4.27; N, 16.46; Found C, 50.74; H, 4.20; N, 16.41.

2.2.22. N⁶-(4-Methylbenzyl)adenosine (11l)—Yield 77%; mp 158–159°C; $[\alpha]_D^{25}$ –67.33 (*c* 0.11, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 22135, pH 2), 269.0 nm (ϵ 22300, pH 7), 269.0 nm (ϵ 22192, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.27 (s, 3H), 3.58 (m, 1H), 3.70 (m, 1H), 3.99 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 4.69 (m, 2H), 5.22 (m, 1H), 5.43 (m, 1H), 5.48 (d, *J* = 5.5 Hz, 1H), 5.92 (d, *J* = 6.5 Hz, 1H), 7.12 (d, *J* = 8.0 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 2H), 8.23 (s, 1H), 8.40 (s, 1H), 8.46 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 21.1, 43.1, 62.2, 71.1, 74.0, 86.4, 88.5, 120.3, 127.6, 129.2, 136.1, 137.5, 140.4, 149.0, 152.8, 155.0. Anal. Calcd for C₁₈H₂₁N₅O₄ requires C, 58.21; H, 5.70; N, 18.86; Found C, 58.43; H, 5.75; N, 18.90.

2.2.23. N⁶-(4-Isopropylbenzyl)adenosine (11m)—Yield 78%; mp 148–150°C; $[\alpha]_D^{25}$ –58.14 (*c* 0.16, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 23879, pH 2), 269.0 nm (ϵ 23535, pH 7), 269.0 nm (ϵ 23078, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.19 (d, *J* = 6.5 Hz, 6H), 2.85 (m, 1H), 3.57 (m, 1H), 3.70 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.64 (m, 1H), 4.69 (m, 2H), 5.22 (m, 1H), 5.42 (m, 1H), 5.47 (d, *J* = 6.5 Hz, 1H), 5.92 (d, *J* = 6.5 Hz, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.28 (d, *J* = 7.5 Hz, 2H), 8.23 (s, 1H), 8.40 (s, 1H), 8.46 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 24.4, 33.6, 43.1, 62.2, 71.1, 74.0, 86.4, 88.5, 120.3, 126.6, 127.7, 137.9, 140.4, 147.3, 149.0, 152.8, 155.0. Anal. Calcd for C₂₀H₂₅N₅O₄ requires C, 60.14; H, 6.31; N, 17.53; Found C, 59.94; H, 6.36; N, 17.51.

2.2.24. N⁶-(4-Vinylbenzyl)adenosine (11n)—Yield 76%; mp 154–156°C; $[\alpha]_D^{25}$ –58.66 (*c* 0.19, MeOH); UV (H₂O) $\lambda_{\max}$ 265.0 nm (ϵ 31103, pH 2), 267.0 nm (ϵ 30473, pH 7), 267.0 nm (ϵ 30601, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.57 (m, 1H), 3.69 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.64 (m, 1H), 4.72 (m, 2H), 5.23 (m, 2H), 5.40 (m, 1H), 5.48 (d, *J* = 5.5 Hz, 1H), 5.79 (d, *J* = 18.0 Hz, 1H), 5.91 (d, *J* = 6.0 Hz, 1H), 6.71 (dd, *J*₁ = 17.5 Hz, *J*₂ = 10.5 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.57 (d, *J* = 8.0 Hz, 2H), 8.23 (s, 1H), 8.41 (s, 1H), 8.51 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 43.2, 62.2, 71.1, 74.0, 86.4, 88.5, 114.2, 120.3, 126.5, 127.9, 136.1, 136.9, 140.3, 140.4, 149.0, 152.8, 155.0. Anal. Calcd for C₁₉H₂₁N₅O₄ requires C, 59.52; H, 5.52; N, 18.27; Found C, 59.45; H, 5.52; N, 18.22.

2.2.25. N⁶-(4-Methoxybenzyl)adenosine (11o)—Yield 78%; mp 147–149°C; $[\alpha]_D^{25}$ –66.51 (*c* 0.12, MeOH); UV (H₂O) $\lambda_{\max}$ 268.0 nm (ϵ 19552, pH 2), 269.0 nm (ϵ 19581, pH 7), 269.0 nm (ϵ 20045, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.70 (m, 1H), 3.73 (s, 3H), 4.00 (m, 1H), 4.18 (m, 1H), 4.66 (m, 3H), 5.23 (m, 1H), 5.44 (m, 1H), 5.49 (d, *J* = 4.5 Hz, 1H), 5.92 (d, *J* = 4.5 Hz, 1H), 6.88 (d, *J* = 7.0 Hz, 2H), 7.30 (d, *J* = 7.0 Hz, 2H), 8.25 (s, 1H), 8.41 (s, 1H), 8.44 (s, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 42.8, 55.5, 62.2, 71.2, 74.0, 86.4, 88.5, 114.1, 120.3, 129.0, 132.5, 140.3, 149.0, 152.8, 155.0, 158.6. Anal. Calcd for C₁₈H₂₁N₅O₅ requires C, 55.81; H, 5.46; N, 18.08; Found C, 56.00; H, 5.37; N, 17.90.

2.2.26. N⁶-(2,4-Difluorobenzyl)adenosine (11p)—Yield 73%; mp 169–171°C; $[\alpha]_D^{25}$ –62.59 (*c* 0.10, MeOH); UV (H₂O) $\lambda_{\max}$ 267.0 nm (ϵ 20041, pH 2), 267.0 nm (ϵ 19901, pH 7), 267.0 nm (ϵ 20057, pH 11); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.59 (m, 1H), 3.71 (m, 1H), 4.00 (m, 1H), 4.18 (m, 1H), 4.65 (m, 1H), 4.74 (m, 2H), 5.23 (m, 1H), 5.39 (m, 1H), 5.49 (d,

$J = 6.0$ Hz, 1H), 5.93 (d, $J = 6.5$ Hz, 1H), 7.03 (m, 1H), 7.24 (m, 1H), 7.40 (m, 1H), 8.25 (s, 1H), 8.44 (s, 1H), 8.49 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 37.0, 62.1, 71.1, 74.0, 86.4, 88.4, 104.0 (t, $J = 25.8$ Hz), 111.7 (dd, $J_1 = 20.8$ Hz, $J_2 = 3.8$ Hz), 120.4, 123.4, 130.7, 140.6, 149.1, 152.8, 154.9, 160.4 (dd, $J_1 = 245.6$ Hz, $J_2 = 12.0$ Hz), 161.7 (dd, $J_1 = 243.4$ Hz, $J_2 = 12.0$ Hz). Anal. Calcd for $\text{C}_{17}\text{H}_{17}\text{F}_2\text{N}_5\text{O}_4$ requires C, 51.91; H, 4.36; N, 17.80; Found C, 51.81; H, 4.35; N, 17.70.

2.2.27. N^6 -(2,4-Dichlorobenzyl)adenosine (11q)—Yield 75%; mp 193–195°C; $[\alpha]^{25}_{\text{D}} -54.59$ (c 0.23, MeOH); UV (H_2O) λ_{max} 267.0 nm (ϵ 17854, pH 2), 267.0 nm (ϵ 15763, pH 7), 267.0 nm (ϵ 17216, pH 11); ^1H NMR (500 MHz, DMSO- d_6) δ 3.57 (m, 1H), 3.69 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.65 (m, 1H), 4.74 (m, 2H), 5.23 (m, 1H), 5.37 (m, 1H), 5.49 (d, $J = 6.0$ Hz, 1H), 5.93 (d, $J = 6.0$ Hz, 1H), 7.29 (m, 1H), 7.38 (m, 1H), 7.65 (m, 1H), 8.23 (s, 1H), 8.46 (s, 1H), 8.55 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 41.2, 62.1, 71.1, 74.0, 86.4, 88.4, 120.4, 127.8, 129.0, 129.8, 132.4, 133.1, 136.4, 140.8, 149.1, 152.9, 154.8. Anal. Calcd for $\text{C}_{17}\text{H}_{17}\text{Cl}_2\text{N}_5\text{O}_4$ requires C, 47.90; H, 4.02; N, 16.43; Found C, 47.80; H, 3.96; N, 16.22.

2.2.28. N^6 -(3,4-Dichlorobenzyl)adenosine (11r)—Yield 73%; mp 184–185°C; $[\alpha]^{24}_{\text{D}} -57.21$ (c 0.19, MeOH); UV (H_2O) λ_{max} 267.0 nm (ϵ 19002, pH 2), 268.0 nm (ϵ 17314, pH 7), 268.0 nm (ϵ 19186, pH 11); ^1H NMR (500 MHz, DMSO- d_6) δ 3.58 (m, 1H), 3.70 (m, 1H), 3.99 (m, 1H), 4.18 (m, 1H), 4.64 (m, 1H), 4.72 (m, 2H), 5.22 (m, 1H), 5.37 (m, 1H), 5.48 (d, $J = 5.5$ Hz, 1H), 5.93 (d, $J = 6.0$ Hz, 1H), 7.35 (d, $J = 7.5$ Hz, 1H), 7.59 (m, 2H), 8.25 (s, 1H), 8.44 (s, 1H), 8.57 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 42.5, 62.1, 71.1, 74.0, 86.4, 88.4, 120.3, 128.0, 129.6, 130.9, 131.3, 140.6, 141.9, 149.1, 152.8, 154.8. Anal. Calcd for $\text{C}_{17}\text{H}_{17}\text{Cl}_2\text{N}_5\text{O}_4$ requires C, 47.90; H, 4.02; N, 16.43; Found C, 48.18; H, 4.06; N, 16.32.

2.2.29. N^6 -(2-Fluoro-4-nitrobenzyl)adenosine (11s)—Yield 68%; mp 147–149°C; $[\alpha]^{25}_{\text{D}} -55.71$ (c 0.17, MeOH); UV (H_2O) λ_{max} 270.0 nm (ϵ 24334, pH 2), 269.0 nm (ϵ 24565, pH 7), 269.0 nm (ϵ 24614, pH 11); ^1H NMR (500 MHz, DMSO- d_6) δ 3.58 (m, 1H), 3.70 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.65 (m, 1H), 4.86 (m, 2H), 5.23 (m, 1H), 5.35 (m, 1H), 5.49 (d, $J = 6.0$ Hz, 1H), 5.93 (d, $J = 6.5$ Hz, 1H), 7.58 (m, 1H), 8.06 (dd, $J_1 = 8.0$ Hz, $J_2 = 2.5$ Hz, 1H), 8.13 (dd, $J_1 = 10.0$ Hz, $J_2 = 2.5$ Hz, 1H), 8.24 (s, 1H), 8.47 (s, 1H), 8.64 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 37.6, 62.1, 71.1, 74.0, 86.4, 88.4, 111.3 (d, $J = 26.8$ Hz), 120.1, 120.4, 130.1, 135.5, 140.8, 147.7 (d, $J = 8.6$ Hz), 149.3, 152.8, 154.8, 159.7 (d, $J = 247.6$ Hz). Anal. Calcd for $\text{C}_{17}\text{H}_{17}\text{FN}_6\text{O}_6$ requires C, 48.57; H, 4.08; N, 19.99; Found C, 48.69; H, 4.01; N, 19.94.

2.2.30. N^6 -(2-Chloro-4-cyanobenzyl)adenosine (11t)—Yield 75%; mp 148–150°C; $[\alpha]^{25}_{\text{D}} -60.56$ (c 0.31, MeOH); UV (H_2O) λ_{max} 268.0 nm (ϵ 24613, pH 2), 267.0 nm (ϵ 24963, pH 7), 267.0 nm (ϵ 25026, pH 11); ^1H NMR (500 MHz, DMSO- d_6) δ 3.58 (m, 1H), 3.69 (m, 1H), 3.99 (m, 1H), 4.17 (m, 1H), 4.65 (m, 1H), 4.81 (m, 2H), 5.24 (m, 1H), 5.35 (m, 1H), 5.49 (d, $J = 6.0$ Hz, 1H), 5.93 (d, $J = 5.0$ Hz, 1H), 7.43 (m, 1H), 7.77 (m, 1H), 8.10 (d, $J = 1.5$ Hz, 1H), 8.23 (s, 1H), 8.48 (s, 1H), 8.63 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 41.8, 62.1, 71.1, 74.0, 86.4, 88.4, 111.6, 118.1, 120.4, 129.2, 131.6, 132.9, 133.0, 140.8, 143.5, 149.3, 152.8, 154.8. Anal. Calcd for $\text{C}_{18}\text{H}_{17}\text{ClN}_6\text{O}_4$ requires C, 51.87; H, 4.11; N, 20.16; Found C, 51.68; H, 4.21; N, 19.93.

2.2.31. N^6 -(2-Fluoro-4-methoxybenzyl)adenosine (11u)—Yield 75%; mp 148–150°C; $[\alpha]^{25}_{\text{D}} -59.04$ (c 0.13, MeOH); UV (H_2O) λ_{max} 268.0 nm (ϵ 23804, pH 2), 268.0 nm (ϵ 23755, pH 7), 268.0 nm (ϵ 23563, pH 11); ^1H NMR (500 MHz, DMSO- d_6) δ 3.57 (m, 1H), 3.68 (m, 1H), 3.75 (s, 3H), 3.99 (m, 1H), 4.17 (m, 1H), 4.64 (m, 1H), 4.69 (m, 2H), 5.23 (m, 1H), 5.40 (m, 1H), 5.48 (d, $J = 5.5$ Hz, 1H), 5.92 (d, $J = 6.0$ Hz, 1H), 6.73 (dd, $J_1 = 8.0$ Hz, $J_2 = 2.5$ Hz, 1H), 6.82 (dd, $J_1 = 12.0$ Hz, $J_2 = 2.5$ Hz, 1H), 7.27 (m, 1H), 8.24 (s, 1H), 8.41 (m, 2H); ^{13}C

NMR (125 MHz, DMSO- d_6) δ 36.9, 56.0, 62.2, 71.1, 74.0, 86.4, 88.4, 101.8 (d, J = 24.9 Hz), 110.5, 118.7 (d, J = 15.6 Hz), 120.3, 130.3, 140.4, 149.0, 152.8, 155.0, 160.1, 161.0 (d, J = 251.8 Hz). Anal. Calcd for $C_{18}H_{20}FN_5O_5 \cdot 0.2H_2O$ requires C, 52.86; H, 5.03; N, 17.12; Found C, 52.85; H, 4.97; N, 17.04.

2.2.32. N^6 -(2,4-Dimethoxybenzyl)adenosine (11v)—Yield 77%; mp 196–198°C; $[\alpha]_D^{25}$ –56.13 (c 0.18, MeOH); UV (H_2O) λ_{max} 267.0 nm (ϵ 20952, pH 2), 270.0 nm (ϵ 21958, pH 7), 270.0 nm (ϵ 21868, pH 11); 1H NMR (500 MHz, DMSO- d_6) δ 3.59 (m, 1H), 3.72 (m, 1H), 3.75 (s, 3H), 3.84 (s, 3H), 4.00 (m, 1H), 4.18 (m, 1H), 4.64 (m, 3H), 5.23 (m, 1H), 5.44 (m, 1H), 5.49 (d, J = 4.0 Hz, 1H), 5.92 (d, J = 4.0 Hz, 1H), 6.45 (d, J = 8.0 Hz, 1H), 6.58 (s, 1H), 7.04 (m, 1H), 8.13 (s, 1H), 8.21 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 38.5, 55.7, 55.9, 62.2, 71.2, 74.0, 86.4, 88.5, 98.7, 104.7, 119.8, 120.4, 128.3, 140.4, 148.9, 152.9, 155.2, 158.1, 160.0. Anal. Calcd for $C_{19}H_{23}N_5O_6$ requires C, 54.67; H, 5.55; N, 16.78; Found C, 54.52; H, 5.54; N, 16.71.

2.3. Molecular modeling

T. gondii adenosine kinase functions by induced fit mechanism and involves a hinge bending motion of two domains. This domain movement is complex, and apparently involves further tuning of the protein structure when substrates bind into the active-site of the enzyme. As a result, our initial modeling attempts of utilizing the *apo* form (1LIO.pdb) [23] of the enzyme were far less successful than subsequent analysis with the *T. gondii* adenosine kinase–adenosine complex (1LII.pdb) [23]. The crystal coordinates of the reported enzyme:substrate complex is missing the following residues: 1–10, 239–240, 255–269, 359–363. Residues 239–240 and 255–269 were added using the SYBYL Biopolymer module (Tripos Associates, St. Louis, MO). The terminal residues were not added because it has been shown by mutation and sequential deletions studies that their effect on the overall binding affinity is negligible. Determination of the binding mode of the N^6 -benzyladenosine analogues was complicated by the fact that the size of the ligand (N^6 -benzyladenosine) is very large as compared to the natural substrate adenosine. N^6 -benzyladenosine analogues are almost ~16 Å in length diagonally, where as the diagonal length of the adenosine is ~8 Å. We are almost doubling the size of the ligand while keeping the size of the pocket the same. Therefore, the induced fit docking method [26] and the prime module of Schrödinger suite [27] were used to judge/perform the protein conformational change according to larger ligand. A second module ‘glide’ was used to dock the ligands in the active site followed by active-site residue modification and refinement by the prime module. After successful active-site modification and refinement by prime and re-docking by glide, enzyme:ligand complexes were subjected to energy minimization [28].

All the calculations were performed on Schrödinger Suite 2006 (Schrödinger Inc.) with GB/SA continuum water solvation model. OPLS_2001 and OPLS_2005 force field was used for induced fit docking and then the same force field was implemented into MACROMODEL 9.1 for energy minimization. For energy minimization studies, residues further away by more than 16 Å from the adenosine binding site were frozen and residues from 6 Å to 16 Å were constrained by harmonic constraints. Only residues inside a 6 Å sphere from the nucleoside were allowed to move freely. EM calculations were performed for 5000 steps or until the energy difference between subsequent structures was 0.05 KJ/mol. In the case of the N^6 -benzyladenosine analogues, the starting conformation was obtained after performing a 5000 step Monte Carlo conformational search under GB/SA continuum water solvation model, Polak-Ribiere Conjugate Gradient (PRCG) as a method for the subsequent energy minimization.

2.4. Evaluation of the *N*⁶-benzyladenosine 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 [19]. 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 MgCl₂, 5 mM creatine phosphate, creatine kinase, 5 μM [8-¹⁴C]adenosine (55 Ci/mol), 50 μL purified *T. gondii* adenosine kinase, prepared as previously described [29] 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 and AMP, 0.17. The amounts of radioactivity in both the substrate and product were calculated on a percentage basis using a computerized Berthold LB-284 Automatic TLC-Linear Analyzers. Apparent *K_i* values of these analogues were calculated from Dixon plots 1/*v* vs. [*I*] by least-squares fitting by computer programs developed by Dr. Naguib as previously described [17–19]. The synthesis of the nucleoside 5prime;-monophosphates from the tested *N*⁶-benzyladenosine analogues was confirmed by HPLC and NMR analyses as previously described [20], indicating that these compounds are alternative substrates to *T. gondii* adenosine kinase. Since these compounds are alternate substrates of *T. gondii* adenosine kinase, their apparent *K_i* values are equal to their apparent *K_m* values [30] as presented in Table 1.

2.5. Evaluation of *N*⁶-benzyladenosine analogues as potential anti-toxoplasma agents against *T. gondii* in tissue culture

The wild type RH and the adenosine kinase deficient mutant *TgAK*⁻³ [22] 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* as was the case *in vitro*. 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 [20, 31]. 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 [20,31]. Briefly, confluent cells (4–5 day incubation) were cultured for 24 hrs in the 24-well flat bottom microtiter plates (~5 x 10⁵/1 mL/well) and incubated at 37°C in 5% CO₂, 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 hr 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 hrs incubation the medium was replaced with 1 mL drug free media containing [5,6-¹³H]uracil (5 μCi/mL) and incubated for another 6 hrs after which the media was removed. The fibroblasts were then released from the wells by trypsinization with the addition of 200 μL trypsin/EDTA (2.5X) 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 pre-washed each with 1 mL double distilled H₂O 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 [19,20,31, 32]. Radiolabel incorporation closely correlates with parasite growth [19,20,31,32].

2.6. Host-toxicity of *N*⁶-benzyladenosine 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 [19,20,31]. Briefly, confluent cells were incubated for at least 24 hrs in 96-well flat bottom microtiterplates (~10⁵/200 µL/well) at 37°C in 5% CO₂, 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 hrs 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 hrs 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

3.1. Chemistry

The substituted benzylamines **3a-b**, **7** and **9** were prepared according to the literature procedures [33,34] as shown in Scheme 1. Compounds **1a-b** were treated with *N*-bromosuccinimide in the presence of benzoyl peroxide to obtain the benzyl bromide derivatives, which were then reacted with di-*tert*-butyl iminodicarboxylate and sodium hydride to give compounds **2a-b**. The Boc groups were removed with 1N HCl/Et₂O in methanol to give the desired benzylamines **3a-b**. Compound **4** was reduced to provide the benzyl alcohol **5**. Bromination of compound **5** with carbon tetrabromide and triphenylphosphine provided the corresponding benzyl bromide followed by introduction of an amino group to give compound **6**. Subsequent deprotection of compound **6** was performed under the acidic conditions described above for compounds **3a-b** to obtain compound **7**. 4-Vinylbenzyl chloride (**8**) was converted into the azide intermediate as a crude oil and treated with triphenylphosphine followed by hydrolysis to yield benzylamine **9**.

*N*⁶-Benzyladenosine analogues **11a-v** were synthesized *via* solution phase parallel synthesis (Scheme 2) [35–37]. Introduction of the appropriate benzylamine was achieved by nucleophilic substitution of commercially available 6-chloropurine riboside (**10**). Compounds **11a-c**, **11e-r** and **11t-v** were obtained in 70–80% yields after purification. However, **11d** and **11s** containing a nitro group were obtained in lower yields. A mild condition (*N,N*-diisopropylethylamine and lower temperature) provided the desired compounds **11d** and **11s** with improved yields.

3.2. Structure-activity relationships by enzyme binding affinity

The binding affinities of *N*⁶-benzyladenosine (**11a**) and its analogues (**11b-v**) to *T. gondii* adenosine kinase were evaluated in enzyme-based assays, along with adenosine as reference substrate (Table 1).

The present results allowed us to examine the relationships between the type and the position of the substitutions on the aromatic ring, as well as the biological activity of these N^6 -benzyladenosine analogues. Chlorine at the *ortho* position (**11c**) exhibited higher binding affinity in comparison to the fluorine substitution (**11b**). Compounds **11e** (*o*-methoxy) and **11g** (*m*-methyl) displayed an increase in the binding affinity to *T. gondii* adenosine kinase when compared to **11d** (*o*-nitro) and **11f** (*m*-fluoro), respectively. It appears that electron-rich substitution at the *ortho* or the *meta* position resulted in an increase in the binding affinity to the enzyme. Our previous studies with 6-benzylthioinosine analogues indicated that substitutions at the *para* position have a substantial impact on the binding affinity to *T. gondii* adenosine kinase [18]. These previous results prompted us to examine the effects of substituents at the *para* position of the benzyl moiety of N^6 -benzyladenosine (**11h-o**). N^6 -(*para*-Methoxybenzyl)adenosine (**11o**) was the best ligand in this series as it produced an 18-fold enhancement in the binding affinity. In the case of disubstituted benzyladenosine analogues (**11p-v**), a small disubstitution such as fluorine (**11p**) appeared to be favored. It was also observed that the difluorinated compound **11p** exhibited improved binding affinity relative to the monofluorinated compounds (**11b**, **11f**, and **11h**).

3.3. Evaluation of anti-toxoplasma activity

N^6 -Benzyladenosine (**11a**) and its analogues (**11b-v**) were evaluated against wild type (RH) and adenosine kinase deficient ($TgAK^{-3}$) strains of *T. gondii* to further investigate their potency as anti-toxoplasma agents (Table 2). Pyrimethamine and sulfadiazine as chemotherapeutic agents for the treatment of toxoplasmosis were used as positive controls.

As shown in Table 2, all of the N^6 -benzyladenosines were effective against infection with the wild type (RH) *T. gondii* in a dose-dependent manner. Furthermore, none of these compounds were found effective against infection with adenosine kinase deficient strain ($TgAK^{-3}$). Therefore, these compounds appear to be the active substrates for *T. gondii* adenosine kinase *in vivo* as was the case *in vitro* (Table 1). Taken as a whole, there was a general trend of correlation between the degree of anti-toxoplasma efficacy in the cell-based activity and the K_m values obtained from the enzyme binding assays (Table 1). As expected, N^6 -(2-methoxybenzyl)adenosine (**11e**) and N^6 -(4-methoxybenzyl)adenosine (**11o**) appeared to be more potent in the cell culture evaluations and were also comparable with that of sulfadiazine and/or pyrimethamine (Table 2). On the other hand, the unsubstituted N^6 -benzyladenosine (**11a**) and N^6 -(2-fluoro-4-nitrobenzyl)adenosine (**11s**), which were weak substrates for *T. gondii* adenosine kinase (Table 1), exhibited lower anti-toxoplasma potency in the cell culture assays (Table 2). Discrepancies in the order of potency between the enzymatic assay (Table 1) and the cell-based assay (Table 2) may be attributed to the differences in cell permeability of these compounds.

We have also tested the host-cell-toxicity of N^6 -benzyladenosine (**11a**) and its analogues (**11b-v**) at concentration up to 50 μ M in an effort to delineate their selective toxicity as potential anti-toxoplasma agents. Most of the N^6 -benzyladenosines had toxic effects on the survival of uninfected host cell (Table 2) and therefore may be inadequate for the treatment of toxoplasmosis. However, N^6 -(2,4-dimethoxybenzyl)adenosine (**11v**) had no detectable host toxicity and hence exhibited favorable potency and selectivity as an anti-toxoplasma agent.

3.4. Molecular modeling

Previous structure-activity relationship studies [17,18] and X-ray structural reports [23,24] revealed the molecular criteria for ligand binding to the adenosine binding site of *T. gondii* adenosine kinase. Recent structural studies of adenosine kinase complex with N^6 -dimethyladenosine and 6-methylmercaptapurine riboside [29] showed the intermediate-conformation between closed (catalytic-conformation) and open lid domain (apo-

conformation), and revealed the conformational steps for catalysis. The molecular recognition analysis from the detailed study of the reported crystal structures of enzyme-ligand complexes [23] and structure-activity relationships analysis [17,18] indicate that the following factors play important roles in ligand binding into the adenosine binding pocket: (a) 2'-OH and 3'-OH are crucial for the binding because they form four strong H-bond with nearby residues, which accounts for a strong electrostatic interaction and conformational determinant for the ribose moiety as shown in Fig. 2a; (b) Active-site map analysis clearly showed the presence of a large hydrophobic regions around the N^6 and N^7 positions of adenine as shown in Fig. 2b; (c) Adenine is surrounded with hydrophobic residues such as Ile22, Leu46, Leu138, Leu142, Tyr169. Our recent modeling and biological studies on 6-benzylthioinosine analogues [18] also revealed that when the substituted benzyl moiety moves into the pocket, the above described hydrophobic residues near the purine motif allow a favorable interaction by the induced fit mechanism and strengthen binding by increasing the van der Waals interaction.

Our study on the determination of binding mode for N^6 -benzyladenosine analogues was complicated because the size of the N^6 -benzyladenosine analogues is almost a twice the size of the natural substrate, adenosine. As *T. gondii* adenosine kinase functions *via* the induced fit mechanism, an advance induced fit docking method [26] was used to allow for the larger ligands that we synthesized in this study. The N^6 -benzyladenosine analogues docked successfully into the *T. gondii* adenosine kinase active-site by induced fit method [27]. The binding mode shows an *anti*-conformation of the nucleoside and an *2'-endo* conformation of the ribose moiety, which are the preferred conformations observed by the co-crystal structure [23]. The benzyl moiety occupies the hydrophobic pocket and forms a favorable van der Waals interaction with surrounding residues. Crucial interactions such as 2'-OH and 3'-OH interactions with Asp24, π - π , and C-H- π (Fig. 2a), which are required for ligand binding into the adenosine pocket, were present for all N^6 -benzyladenosine analogues while significant differences in van der Waals interactions were observed for individual compounds. These results indicate that the type and position of the substituents on the benzyl group of the N^6 -benzyladenosines significantly affect the van der Waals interaction and ultimately lead to differences in their binding affinities.

Fig. 3 shows the molecular surface around adenosine (natural substrate), N^6 -(4-methoxybenzyl)adenosine (**11o**), and N^6 -(2,4-dimethoxybenzyl)adenosine (**11v**), the best ligands among the synthesized N^6 -benzyladenosine analogues, after induced fit modeling, which explains the reason for accommodating the larger ligands in the adenosine binding site of *T. gondii* adenosine kinase by conformational changes in nearby hydrophobic residues. In the case of N^6 -(4-methoxybenzyl)adenosine (**11o**), the benzyl group position in the hydrophobic pocket and the presence of methoxy substitution at the *para* position increases the van der Waals surface interaction with Leu46. On the other hand, N^6 -(2,4-dimethoxybenzyl)adenosine (**11v**) showed the orientation of additional *ortho*-methoxy substituent towards middle of the Thr140 and Leu142 residue. Fig. 4 shows the binding modes of N^6 -(4-chlorobenzyl)adenosine (**11i**) and N^6 -(4-methoxybenzyl)adenosine (**11o**), where N^6 -(4-chlorobenzyl)adenosine (**11i**) exhibits less van der Waals surface interaction with Leu46 than N^6 -(4-methoxybenzyl)adenosine (**11o**). The present results suggest the long tubular hydrophobic region at the N^6 region can accommodate the benzyl group and in spite of similar binding mode, the benzyl substituents play an important role to induce the favorable or unfavorable van der Waals interaction with the surrounding residues.

4. Discussion

N^6 -benzyladenosine analogues were synthesized and evaluated for their binding affinity to purified *T. gondii* adenosine kinase. Compound **11a**, containing unsubstituted benzyl moiety, showed a weak binding affinity with a K_m value of 179.8 μ M to *T. gondii* adenosine kinase.

Compounds **11c**, **11e**, **11o**, and **11p** were the best among the synthesized analogues ($K_m = 12.0$, 11.2, 9.9, and 12.1 μM , respectively), and provided a 15–18-fold increase in the binding affinity relative to the unsubstituted N^6 -benzyladenosine (**11a**). Furthermore, the anti-toxoplasma efficacy and host toxicity of these compounds were tested in cell culture. Varying the type and position of the substituents on the aromatic ring led to different degrees of potency and selectivity as anti-toxoplasma agents. However, most of the N^6 -benzyladenosine, in contrast to their 6-benzylinosine counterparts [20], were toxic to the host and therefore, are may be inadequate for the treatment of toxoplasmosis. In spite of the host-toxicity of most of the synthesized N^6 -benzyladenosine analogues, N^6 -(2,4-dimethoxybenzyl)adenosine (**11v**) exhibited favorable anti-toxoplasma activity without host-toxicity. These results demonstrate the necessity of testing and evaluating newly synthesized analogues irrespective of their structural similarity to promising analogues before a general conclusion can be made.

The enhanced binding of the synthesized N^6 -benzyladenosine analogue was due mainly to the hydrophobic effect mediated by hydrophobic residues (Y169, F201, L46, and L142) to gain favorable van der Waals interactions around benzyl moiety of the ligands, as established by molecular modeling studies. In addition, we analyzed the MBAE (multi-ligand bimolecular association with energetics) calculation of N^6 -(4-chlorobenzyl)adenosine (**11i**), N^6 -(4-methoxybenzyl)adenosine (**11o**), and N^6 -(2,4-dimethoxybenzyl)adenosine (**11v**) (Table 3) to determine whether or not differences in the binding energies of these compounds may also be involved in enhancing their binding with the enzyme. Compounds **11o** and **11v** gained favorable van der Waals interaction (–36.1 kcal/mol and –44.2 kcal/mol, respectively), which led to reduced total binding energy difference by –34.4 kcal/mol and –27.1 kcal/mol, respectively. In contrast, compound **11i** had less favorable van der Waals interaction (9.2 kcal/mol) as well as an increased binding energy difference of 15.2 kcal/mol. These results indicate that differences in the binding energies could also contribute to the differences in the binding affinities of these compounds to *T. gondii* adenosine kinase

In summary, N^6 -benzyladenosine and its analogues were synthesized as a novel class of anti-toxoplasma agents. Molecular modeling studies were conducted to understand their binding mode to *T. gondii* adenosine kinase. Varying the type and position of the substituents on the aromatic ring resulted in different degrees of the binding affinity to *T. gondii* adenosine kinase as well as potency and selectivity as anti-toxoplasma agents. Based on their structure-activity relationships, additional syntheses of various other analogues as well as biological studies are warranted.

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References

1. Lane HC, Laughon BE, Falloon J, Kovacs JA, Davey RT Jr, Polis MA, Masur H. Recent advances in the management of AIDS-related opportunistic infections. *Ann Intern Med* 1994;120:945–55. [PubMed: 7909657]
2. Klepser ME, Lepser TB. Drug treatment of HIV-related opportunistic infections. *Drugs* 1997;53:40–73. [PubMed: 9010648]
3. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* 2004;363:1965–76. [PubMed: 15194258]
4. Schürmann D, Bergmann F, Albrecht H, Padberg J, Wünsche T, Grünwald T, Schürmann M, Grobusch M, Vallée M, Ruf B, Suttrop N. Effectiveness of twice-weekly pyrimethamine-sulfadoxine as primary prophylaxis of *pneumocystis carinii* pneumonia and toxoplasmic encephalitis in patients with advanced HIV infection. *Eur J Clin Microbiol Infect Dis* 2002;21:353–61. [PubMed: 12072919]

5. Petersen E, Schmidt DR. Sulfadiazine and pyrimethamine in the postnatal treatment of congenital toxoplasmosis: what are the options? *Expert Rev Anti-infect Ther* 2003;1:175–82. [PubMed: 15482110]
6. Wong S, Remington JS. Biology of *Toxoplasma gondii*. *AIDS* 1993;7:299–316. [PubMed: 8471191]
7. Chio LC, Queener SF. Identification of highly potent and selective inhibitor of *Toxoplasma gondii* dihydrofolate reductase. *Antimicrob Agents Chemother* 1993;37:1914–23. [PubMed: 8239605]
8. Antinori A, Murri R, Ammassari A, de Luca A, Linzalone A, Cingolani A, Damiano F, Mauro G, Vecchiet J, Scoppettuolo G. Aerosolized pentamidine, cotrimoxazole and dapsone-pyrimethamine for primary prophylaxis of *pneumocystis carinii* pneumonia and toxoplasmic encephalitis. *AIDS* 1995;9:1343–50. [PubMed: 8605054]
9. Croft SL. The current status of antiparasite chemotherapy. *Parasitology* 1997;114:S3–S15. [PubMed: 9309765]
10. Joss, AWL. Human toxoplasmosis. Ho-Yen, DO.; Joss, AWL., editors. New York: Oxford University Press; 1992. p. 120–5.
11. Subauste CS, Remington JS. Immunity to *Toxoplasma gondii*. *Curr Opin Immunol* 1993;5:532–7. [PubMed: 8216929]
12. el Kouni MH. Potential chemotherapeutic targets in the purine metabolism of parasites. *Pharmacol Ther* 2003;99:283–309. [PubMed: 12951162]
13. el Kouni MH. Adenosine metabolism in *Toxoplasma gondii*: potential targets for chemotherapy. *Curr Pharm Des.* in press
14. Pfefferkorn ER, Pfefferkorn LC. The biochemical basis for resistance to adenine arabinoside in a mutant of *Toxoplasma gondii*. *J Parasitol* 1978;64:486–92. [PubMed: 207846]
15. Krug EC, Marr JJ, Berens RL. Purine metabolism in *Toxoplasma gondii*. *J Biol Chem* 1989;264:10601–7. [PubMed: 2732241]
16. Chaudhary K, Darling JA, Fohl LM, Sullivan WJ Jr, Donald RGK, Pfefferkorn ER, Ullman B, Roos DS. Purine salvage pathways in the apicomplexan parasite *Toxoplasma gondii*. *J Biol Chem* 2004;279:31221–7. [PubMed: 15140885]
17. Iltzsch MH, Uber SS, Tankersley KO, el Kouni MH. Structure activity relationship for the binding of nucleoside ligands to adenosine kinase from *Toxoplasma gondii*. *Biochem Pharmacol* 1995;49:1501–12. [PubMed: 7763293]
18. Yadav V, Chu CK, Rais RH, Al Safarjalani ON, Guarcello V, Naguib FNM, el Kouni MH. Synthesis, biological activity and molecular modeling of 6-benzylthioinosine analogues as subversive substrates of *Toxoplasma gondii* adenosine kinase. *J Med Chem* 2004;47:1987–96. [PubMed: 15055998]
19. el Kouni MH, Guarcello V, Al Safarjalani ON, Naguib FNM. Metabolism and selective toxicity of 6-nitrobenzylthioinosine in *Toxoplasma gondii*. *Antimicrob Agents Chemother* 1999;43:2437–43. [PubMed: 10508021]
20. Rais RH, Al Safarjalani ON, Yadav V, Guarcello V, Kirk M, Chu CK, Naguib FNM, el Kouni MH. 6-Benzylthioinosine analogues as subversive substrate of *Toxoplasma gondii* adenosine kinase: activities and selective toxicities. *Biochem Pharmacol* 2005;69:1409–19. [PubMed: 15857605]
21. Gupte A, Buolamwini JK, Yadav V, Chu CK, Naguib FNM, el Kouni MH. 6-Benzylthioinosine analogues: promising anti-toxoplasmic agents as inhibitors of the mammalian nucleoside transporter ENT1 (es). *Biochem Pharmacol* 2005;71:69–73. [PubMed: 16310172]
22. Sullivan WJ, Chiang CW, Wilson CM, Naguib FNM, el Kouni MH, Donald RGK, Roos DS. Insertional tagging and cloning of at least two loci associated with resistance to adenine arabinoside in *Toxoplasma gondii* and cloning of the adenosine kinase locus. *Mol Biochem Parasitol* 1999;103:1–14. [PubMed: 10514076]
23. Schumacher MA, Scott DM, Mathews II, Ealick SE, Roos DS, Ullman B, Brennan RG. Crystal structure of *Toxoplasma gondii* adenosine kinase reveal a novel catalytic mechanism and prodrug binding. *J Mol Biol* 2000;298:875–93. [PubMed: 10801355]
24. Zhang Y, el Kouni MH, Ealick SE. Structure of *Toxoplasma gondii* adenosine kinase in complex with an ATP analog at 1.1 Å resolution. *Acta Cryst D* 2006;D62:142–5.
25. Mathews II, Erion MD, Ealick SE. Structure of human adenosine kinase at 1.5 Å resolution. *Biochemistry* 1998;37:15607–15620. [PubMed: 9843365]

26. Sherman W, Day T, Jacobson MP, Friesner RA, Farid R. Novel procedure for modeling ligand/receptor induced fit effects. *J Med Chem* 2006;49:534–53. [PubMed: 16420040]
27. Schrödinger Suite 2006 Induced Fit Docking Protocol; Glide version 4.0, Schrödinger, LLC, New York, NY, 2005; Prime version 1.5, Schrödinger, LLC, New York, NY, 2005.
28. MacroModel, version 9.1, Schrödinger, LLC, New York, NY, 2005.
29. Zhang Y, el Kouni MH, Ealick SE. Substrate analogs induced intermediate conformational changes in *Toxoplasma gondii* adenosine kinase. *Acta Cryst.* in Press
30. Cha S. Kinetics of enzyme reactions with competing alternative substrates. *Mol Pharmacol* 1968;4:621–9. [PubMed: 5725576]
31. Al Safarjalani ON, Naguib FNM, el Kouni MH. Uptake of nitrobenzylthioinosine and purine β -L-nucleosides by intracellular *toxoplasma gondii*. *Antimicrob Agents Chemother* 2003;47:3247–51. [PubMed: 14506037]
32. Pfefferkorn ER, Pfefferkorn LC. Specific labeling of intracellular *toxoplasma gondii* with uracil. *J Protozool* 1977;24:449–53. [PubMed: 21288]
33. Schacht, AL.; Smith, GF.; Wiley, MR. Antithrombotic agents. US Patent 5710130. 1998.
34. Lee K, Park CW, Jung WH, Park HD, Lee SH, Chung KH, Park SK, Kwon OH, Kang M, Park DH, Lee SK, Kim EE, Yoon SK, Kim A. Efficacious and orally bioavailable thrombin inhibitors based on a 2,5-thienylamidine at the P1 position: discovery of *N*-carboxymethyl-*D*-diphenylalanyl-*L*-prolyl [(5-amidino-2-thienyl)methyl]amide. *J Med Chem* 2003;46:3612–22. [PubMed: 12904065]
35. An H, Cook PD. Methodologies for generating solution-phase combinatorial libraries. *Chem Rev* 2000;100:3311–40. [PubMed: 11777426]
36. Kusachi S, Thompson RD, Yamada N, Daly DT, Olsson RA. Dog coronary artery adenosine receptor: structure of the N^6 -aryl subregion. *J Med Chem* 1986;29:989–96. [PubMed: 3012086]
37. Bressi JC, Choe J, Hough MT, Buckner FS, Van Voorhis WC, Verlinde CLMJ, Hol WGJ, Gelb MH. Adenosine analogues as inhibitors of *trypanosoma brucei* phosphoglycerate kinase: elucidation of a novel binding mode for a 2-amino- N^6 -substituted adenosine. *J Med Chem* 2000;43:4135–50. [PubMed: 11063610]

Fig 1.

(a) Superimposition of the apo *T. gondii* adenosine kinase (yellow) and enzyme:adenosine:ATP complex (green) highlights the 30° domain rotation of lid domain (upper small domain) towards large domain near to substrate binding site (space filling representation for substrates). (b) Catalytic site showing the mode of binding for the transfer of γ -phosphate from ATP to adenosine mediated by Arg136 interactions.

Fig 2.

(a) Binding mode of adenosine to *T. gondii* adenosine kinase with hydrophilic (yellow dotted H-bonds) and hydrophobic effects (green mesh): H-bonds by 2'-OH and 3'-OH groups of adenosine with Asp24, Gly69, and Asn73 residues are represented by the yellow dotted line. Hydrophobic stacking by adenine with Tyr169 and Ile22 by π - π and C-H- π interaction, other hydrophobic effect by Leu46 and Leu142 are represented by the green mesh. (b) Active-site map analysis of enzyme shows the hydrophobic pocket (brown patch) near to N^6 -region of adenine, the hydrophilic region of enzyme (white patch), adenine occupying the hydrophobic region (brown patch), and the ribose moiety in the hydrophilic region (white patch).

Fig 3.

(a) Binding mode of the natural substrate, adenosine to *T. gondii* adenosine kinase. (b) Binding mode of *N*⁶-(4-methoxybenzyl)adenosine (**11o**) showing how the induced fit mechanism is working to accommodate a larger ligand in the active site of *T. gondii* adenosine kinase after conformational changes of nearby hydrophobic residues. (c) Binding mode of *N*⁶-(2,4-dimethoxybenzyl)adenosine (**11v**) showing the orientation of additional 2-methoxy substituent towards middle of the Thr140 and Leu142 residues.

Fig 4.

(a) Superimposed structure of the strong substrate *N*⁶-(4-methoxybenzyl)adenosine (**11o**) and the weaker substrate *N*⁶-(4-chlorobenzyl)adenosine (**11i**) showing the same binding mode with the nearby residues in the active site of *T. gondii* adenosine kinase. (b) The binding mode of **11i** to *T. gondii* adenosine kinase showing a low van der Waals surface area for hydrophobic effect with Leu46. (c) While more surface area is found in the case of **11o** for hydrophobic effect with Leu46 residue.

Scheme 1.

Synthesis of substituted benzylamines **3a-b**, **7** and **9**. (a) N-bromosuccinimide, CCl₄, benzoyl peroxide, reflux, 8 h; (b) NH(Boc)₂, NaH, THF, rt, 7 h; (c) 1N HCl/Et₂O, MeOH, rt, 5 h; (d) NaBH₄, MeOH, rt, 3 h; (e) CBr₄, PPh₃, CH₂Cl₂, rt, 3 h; (f) NaN₃, DMF, rt, 1 h; (g) PPh₃, H₂O, benzene, 70°C, 3 h.

Scheme 2.

Synthesis of *N*⁶-benzyladenosine analogues **11a-v**. (a) appropriate benzylamine, TEA, *n*-PrOH, 100°C, 11 h; (b) 2-nitrobenzylamine or 2-fluoro-4-nitrobenzylamine, DIEA, 80°C, 11 h.

Table 1Binding affinities (apparent K_m) of *N*6-benzyladenosine and its analogues to *Toxoplasma gondii* adenosine kinase

Compound	R ₁ (ortho)	R ₂ (meta)	R ₃ (para)	K_m [μ M] ^a
11a <i>N</i> ⁶ -benzyladenosine	H	H	H	179.8 ± 18.8
11b <i>N</i> ⁶ -(2-Fluorobenzyl)adenosine	F	H	H	51.3 ± 10.5
11c <i>N</i> ⁶ -(2-Chlorobenzyl)adenosine	Cl	H	H	12.0 ± 2.4
11d <i>N</i> ⁶ -(2-Nitrobenzyl)adenosine	NO ₂	H	H	37.4 ± 6.6
11e <i>N</i> ⁶ -(2-Methoxybenzyl)adenosine	OCH ₃	H	H	11.2 ± 1.8
11f <i>N</i> ⁶ -(3-Fluorobenzyl)adenosine	H	F	H	82.5 ± 14.4
11g <i>N</i> ⁶ -(3-Methylbenzyl)adenosine	H	CH ₃	H	25.3 ± 4.3
11h <i>N</i> ⁶ -(4-Fluorobenzyl)adenosine	H	H	F	52.6 ± 7.8
11i <i>N</i> ⁶ -(4-Chlorobenzyl)adenosine	H	H	Cl	81.2 ± 11.4
11j <i>N</i> ⁶ -(4-Cyanobenzyl)adenosine	H	H	CN	17.9 ± 4.8
11k <i>N</i> ⁶ -(4-Trifluoromethylbenzyl)adenosine	H	H	CF ₃	98.7 ± 19.3
11l <i>N</i> ⁶ -(4-Methylbenzyl)adenosine	H	H	CH ₃	27.0 ± 4.7
11m <i>N</i> ⁶ -(4-Isopropylbenzyl)adenosine	H	H	ⁱ Pr	47.1 ± 7.9
11n <i>N</i> ⁶ -(4-Vinylbenzyl)adenosine	H	H	CH=CH ₂	88.7 ± 13.4
11o <i>N</i> ⁶ -(4-Methoxybenzyl)adenosine	H	H	OCH ₃	9.9 ± 2.2
11p <i>N</i> ⁶ -(2,4-Difluorobenzyl)adenosine	F	H	F	12.1 ± 2.0
11q <i>N</i> ⁶ -(2,4-Dichlorobenzyl)adenosine	Cl	H	Cl	20.2 ± 6.5
11r <i>N</i> ⁶ -(3,4-Dichlorobenzyl)adenosine	H	Cl	Cl	35.8 ± 3.2
11s <i>N</i> ⁶ -(2-Fluoro-4-nitrobenzyl)adenosine	F	H	NO ₂	167.8 ± 25.3
11t <i>N</i> ⁶ -(2-Chloro-4-cyanobenzyl)adenosine	Cl	H	CN	53.7 ± 13.6
11u <i>N</i> ⁶ -(2-Fluoro-4-methoxybenzyl)adenosine	F	H	OCH ₃	25.5 ± 3.6
11v <i>N</i> ⁶ -(2,4-Dimethoxybenzyl)adenosine	OCH ₃	H	OCH ₃	27.6 ± 1.7
Adenosine	-	-	-	5.2 ± 0.3

^a All values represent the mean ± SD of at least 2 independent experiments with 3 replica each as previously described [17–20].

Table 2

The effect of different doses of *N*⁶-benzyladenosine and its analogues on host-toxicity^a and percent survival^b of wild type and adenosine kinase deficient (*TgAK*⁻³) strains of *Toxoplasma gondii* grown in human fibroblasts in culture

Compound	Infection	Concentration (μM)					IC ₅₀ (μM)
		0	5	10	25	50	
11a <i>N</i> ⁶ -benzyladenosine	wild type (RH) <i>TgAK</i> ⁻³	100	99.2	91.1	82.2	73.1	791 ± 53
	none ^a	100	22.9	20.9	19.1	18.0	
11b <i>N</i> ⁶ -(2-Fluorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	98.3	89.5	36.0	20.4	37.8 ± 27.9
	none	100	100	100	100	100	
11c <i>N</i> ⁶ -(2-Chlorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	79.3	78.1	68.1	64.7	10.6 ± 1.7
	none	100	100	100	100	100	
11d <i>N</i> ⁶ -(2-Nitrobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	74.0	71.2	57.6	57.2	15.0 ± 0.9
	none	100	100	100	100	100	
11e <i>N</i> ⁶ -(2-Methoxybenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	75.2	22.1	0.2	0.0	8.7 ± 3.2
	none	100	100	100	100	100	
11f <i>N</i> ⁶ -(3-Fluorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	26.8	22.6	22.3	21.4	23.2 ± 3.7
	none	100	93.6	75.2	45.7	24.7	
11g <i>N</i> ⁶ -(3-Methylbenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	100	100	100	100	8.5 ± 0.3
	none	100	70.5	28.2	3.5	0.96	
11h <i>N</i> ⁶ -(4-Fluorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	100	95.5	89.5	87.6	20.6 ± 2.9
	none	100	96.3	92.5	33.9	19.9	
11i <i>N</i> ⁶ -(4-Chlorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	44.1	33.8	15.7	14.6	25.8 ± 1.3
	none	100	91.4	80.1	44.1	32.5	
11j <i>N</i> ⁶ -(4-Cyanobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	100	100	100	78.5	8.3 ± 1.2
	none	100	72.5	16.7	2.2	0.0	
11k <i>N</i> ⁶ -(4-Trifluoromethylbenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	55.7	53.7	45.8	31.9	22.9 ± 2.9
	none	100	93.0	75.0	42.0	26.3	
11l <i>N</i> ⁶ -(4-Methylbenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	100	100	100	100	10.5 ± 1.6
	none	100	83.8	33.0	4.3	1.4	
11m <i>N</i> ⁶ -(4-Isopropylbenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	86.7	83.4	80.5	74.2	22.0 ± 2.4
	none	100	96.2	88.5	38.9	22.0	
11n <i>N</i> ⁶ -(4-Vinylbenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	100	100	100	100	26.2 ± 1.9
	none	100	88.1	83.0	50.5	27.4	
11o <i>N</i> ⁶ -(4-Methoxybenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	98.9	89.3	84.6	76.5	7.5 ± 1.0
	none	100	66.9	16.9	1.9	0.0	
11p <i>N</i> ⁶ -(2,4-Difluorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³	100	93.4	90.5	72.3	59.7	8.2 ± 2.3
	none	100	67.7	33.6	2.4	0.0	
	<i>TgAK</i> ⁻³	100	100	100	100	100	
	none	100	69.6	63.5	58.8	40.5	

Compound	Infection	Concentration (μM)					IC ₅₀ (μM)
		0	5	10	25	50	
11q <i>N</i> ⁶ -(2,4-Dichlorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	78.3 100 100	27.4 100 100	1.8 100 100	0.0 100 100	9.3 ± 0.8
11r <i>N</i> ⁶ -(3,4-Dichlorobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	85.0 100 100	73.3 100 100	11.8 100 100	2.7 100 100	14.3 ± 1.2
11s <i>N</i> ⁶ -(2-Fluoro-4-nitrobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	97.8 100 100	91.0 100 100	75.5 100 100	66.4 100 100	230 ± 23
11t <i>N</i> ⁶ -(2-Chloro-4-cyanobenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	99.2 100 100	96.3 100 100	32.3 100 100	21.2 100 100	21.1 ± 1.7
11u <i>N</i> ⁶ -(2-Fluoro-4-methoxybenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	73.4 100 100	39.0 100 100	6.7 100 100	1.1 100 100	9.5 ± 0.9
11v <i>N</i> ⁶ -(2,4-Dimethoxybenzyl)adenosine	wild type (RH) <i>TgAK</i> ⁻³ none	100 100 100	72.0 100 100	29.9 100 100	3.7 100 100	0.5 100 100	8.7 ± 0.6
pyrimethamine	wild type (RH) none	100 100	98.4 54.8	100 36.3	100 13.8	100 5.9	5.3 ± 1.0
sulfadiazine	wild type (RH) none	100 100	75.7 98.2	64.5 99.7	59.2 99.8	46.7 102.5	7.0 ± 1.9

^a Host toxicity of uninfected cells was measured by MTT method in at least 2 independent experiments each of 3 replica as previously described [19,20,31].

^b Percent survival of parasites was measured by incorporation of [5,6-¹³H]uracil in at least 2 independent experiments of 3 replica each as previously described [19,20,31].

Table 3

Comparison between MBAE (multi-ligand bimolecular association with energetics) calculation of representative *N*⁶-benzyladenosine analogues after induced fit docking and energy minimization.

Compound	<i>K_m</i> [μM]	Complex total energy(kcal/mol)	Electrostatic	Energy difference results ΔE: kcal / mol VdW ^a	Total
11i <i>N</i> ⁶ -(4-Chlorobenzyl)adenosine	81.2 ± 11.4	-6279.6	-52.1	9.2	15.2
11o <i>N</i> ⁶ -(4-Methoxybenzyl)adenosine	9.9 ± 2.2	-6331.5	-86.1	-36.1	-34.4
11v <i>N</i> ⁶ -(2,4-Dimethoxybenzyl)adenosine	27.6 ± 1.7	-6317.2	-53.7	-44.2	-27.1

^a van der Waals interaction.