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Structure–activity relationship for nucleoside analogs as inhibitors or substrates of adenosine kinase from *Mycobacterium tuberculosis* I. Modifications to the adenine moiety

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Ade, adenine
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Ado kinase, adenosine kinase
BSA, bovine serum albumin
DTT, dithiothreitol
formycin A, 8-aza-9-deaza-Ado
methyl-Ado, 2-methyl-adenosine
MIC, minimum inhibitory concentration
SAR, structure–activity relationship

ABSTRACT

Adenosine kinase (Ado kinase, EC 2.7.1.20) is a purine salvage enzyme that phosphorylates adenosine (Ado) to AMP. Ado kinase from *Mycobacterium tuberculosis* also catalyzes an essential step in the conversion of 2-methyl-Ado to a compound with selective antimycobacterial activity. In order to aid in the design of more potent and selective Ado analogs, eighty nucleoside analogs with modifications to the adenine (Ade) moiety of Ado were evaluated as both substrates and inhibitors of Ado kinase from *M. tuberculosis*, and a subset was further tested with human Ado kinase for the sake of comparison. The best substrates were 2-aza-Ado, 8-aza-9-deaza-Ado, and 2-fluoro-Ado and the most potent inhibitors were N^1-benzyl-Ado (K_i = 0.19 μM), 2-fluoro-Ado (K_i = 0.5 μM), 6-cyclopentyloxy-purine riboside (K_i = 0.15 μM), and 7-iodo-7-deaza-Ado (K_i = 0.21 μM). These studies revealed the presence of a hydrophobic pocket near the N^6- and N^1-positions that can accommodate substitutions at least as large as a benzyl group. The ability to fit into this pocket increased the likelihood that a compound would be an inhibitor and not a substrate. The 2-position was able to accommodate exocyclic substitutions as large as a methoxy group, although substrate activity was low. Similarly, the 7-position could bind an exocyclic group as large as a carboxamido moiety. However, all of the compounds tested with modifications at the 7-position were much better inhibitors than substrates. MIC studies performed with selected compounds have yielded several Ado analogs with promising antitubercular activity. Future studies will utilize this information for the design of new analogs that may be selective antitubercular agents.

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1. Introduction

Tuberculosis is a global health problem, currently infecting over one-third of the world's population and resulting in over 2-million deaths annually [1–3]. Although the incidence of tuberculosis (TB) declined for decades due to effective drug treatment, the emergence of drug-resistant strains of tuberculosis and the advent of HIV-TB co-infection has led to a global increase in TB-related morbidity and mortality [3]. Short-course treatment regimens for TB includes at least three drugs taken over a period of 6–9 months [1]. Failure to adhere to the treatment regimen due to the long duration and complexity of treatment is associated with development of antibiotic-resistant strains of *Mycobacterium tuberculosis* [4,5]. New antitubercular drugs are needed to treat antibiotic-resistant strains of *M. tuberculosis* and simplify the current treatment regimen [1,3,5]. The scope and severity of TB infection warrants the development of antimicrobials specifically for use against *M. tuberculosis*.

The tuberculosis antimicrobial acquisition and coordination facility (TAACF) was created in order to seek out and develop novel antitubercular drugs [6]. Ideally, new drugs would have a mechanism of action that differs from existing therapies and would be effective against latent TB infection [4]. 2-Methyl-adenosine (methyl-Ado) was one of several adenosine (Ado) analogs identified by the TAACF with promising antitubercular activity. With an MIC of 4 μg/ml and an IC_{50} value of 80 μg/ml in CEM cells [7], methyl-Ado exhibited both potency and selectivity for *M. tuberculosis*. This compound has also demonstrated antitubercular activity in a hypoxic downshift model of latent infection [7]. Although the mechanism of action has not yet been fully elucidated, methyl-Ado is a nucleoside analog, and as such is likely to have a mechanism of action that differs from current TB therapies. Nucleoside analogs have a history of efficacy and safety as antiviral and anticancer agents. While nucleoside analogs are attractive for drug development, there are also obstacles to be considered in their development. Our goal is to identify compounds that can be activated by *M. tuberculosis* enzymes but do not interact with enzymes involved in human purine metabolism.

In vitro metabolism studies were performed with *M. tuberculosis* H37Ra in an effort to understand the mechanism of action of methyl-Ado. These studies revealed that methyl-AMP comprises 99% of the intracellular metabolites of methyl-Ado [8]. Methyl-Ado could be converted to methyl-AMP via several different pathways including direct phosphorylation by adenosine kinase (Ado kinase), cleavage to adenine followed by phosphoribosylation by adenine phosphoribosyl transferase, or a multistep pathway involving adenosine deaminase (Ado deaminase) plus several other enzymes (Fig. 1). However, Chen et al. [9] determined that Ado kinase was responsible for the phosphorylation of methyl-Ado by demonstrating that methyl-Ado-resistant strains of *M. tuberculosis* were deficient in Ado kinase. Furthermore, when the gene that codes for Ado kinase (*adoK*, Rv 2202c, GenBank accession no. NP216718) was cloned back into methyl-Ado-resistant strains of *M. tuberculosis*, sensitivity of the bacteria to methyl-Ado was restored [10]. These studies confirmed that methyl-Ado elicited its effect in an Ado kinase-dependent manner.

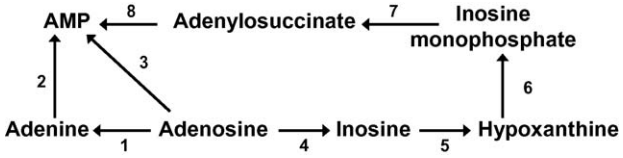

Fig. 1 – Enzymes involved in the conversion of Ado to AMP. (1) Ado hydrolase, (2) adenine phosphoribosyl transferase, (3) Ado kinase, (4) Ado deaminase, (5) purine nucleoside phosphorylase, (6) hypoxanthine–guanine phosphoribosyl transferase, (7) adenylosuccinate synthase, and (8) adenylosuccinate lyase.

Ado kinase (EC 2.7.1.20) is a purine salvage enzyme that catalyzes the phosphorylation of Ado to AMP. Ado kinase is a member of the PfkB family of carbohydrate and nucleoside kinases, a family that includes ribokinase, fructokinase, and hexokinase among its members [11]. This enzyme is found ubiquitously in eukaryotes, plants, fungi, and parasites, but is seldom found in prokaryotes. To the best of our knowledge, *M. tuberculosis* is the first bacterium in which Ado kinase activity has been characterized and the gene positively identified [10,12]. Biochemical characterization of *M. tuberculosis* Ado kinase revealed that this enzyme has properties that are unique from other known Ado kinases. With at most 24% homology with known Ado kinases, *M. tuberculosis* Ado kinase had a primary structure that prevented its positive identification. This enzyme was more similar to ribokinases than other Ado kinases in terms of primary structure, quaternary structure, and stimulation by monovalent cations [10,11,13,14]. This similarity was confirmed when phylogenetic analysis of known Ado kinases, ribokinases, and other members of the PfkB family revealed that this enzyme was evolutionarily more related to ribokinases than other Ado kinases [10]. Furthermore, differences in the K_i for iodotubercidin and the K_m and V_{max} for Ado, ATP, and methyl-Ado suggested that there are significant differences in the active site of *M. tuberculosis* Ado kinase that may be exploited for drug development [10].

Since Ado kinase is central for the mechanism of methyl-Ado and several other Ado analogs [8,9], knowledge about the active site of this enzyme will be a useful resource for design of other nucleoside analogs. It is possible that Ado kinase can be exploited for drug development in two ways. First, it can provide the activating step for subversive substrates to be phosphorylated to toxic metabolites that exert their effect downstream, as in the case of methyl-Ado. Second, if this enzyme is demonstrated to be necessary for growth and survival of *M. tuberculosis* in vivo, then Ado kinase will also be targeted for development of specific inhibitors. This work describes the evaluation of 80 nucleoside analogs as both substrates for Ado kinase and inhibitors of this enzyme for the purpose of gaining a thorough understanding of the requirements for binding of an Ado analog to Ado kinase. Since new compounds should be metabolized by *M. tuberculosis* but not human enzymes, we have included a comparison with human Ado kinase for compounds that were either good substrates or inhibitors of *M. tuberculosis* Ado kinase.

2. Materials and methods

2.1. Chemicals

The nucleoside analogs used in this study were obtained from several different sources (Table 1). Ado (**1**), 9-[β-D-ribofuranosyl]-purine (**24**), 6-chloro-purine riboside (**25**), 6-oxy-purine riboside (**28**), 6-mercapto-purine riboside (**29**), 6-methoxy-purine riboside (**32**), 8-bromo-Ado (**54**), and 8-aza-9-deaza-Ado (**55**) were purchased from Sigma-Aldrich (St. Louis, MO). 6-Bromo-purine riboside (**26**), 6-iodo-purine riboside (**27**), 8-azido-Ado (**52**), 6-nitrobenzyl-mercapto-purine riboside (**35**), 6-benzyl-mercapto-purine riboside (**36**) and 9-deaza-Ado (**67**) were kindly provided by Dr. Mahmoud el Kouni (University of Alabama at Birmingham, Birmingham, AL). 2-Fluoro-3-deaza-Ado (**22**) was a gift from Dr. Alan C. Sartorelli (Yale University, New Haven, CT). The National Institutes of Health (Bethesda, MD) provided 2-β-D-ribofuranosylthiazole-4-carboxamide

Table 1 – Ado kinase activity in the presence of Ado analogs with modifications to the adenine moiety

	Compound name	*M. tuberculosis* specific activity (nmol/mg min)	Inhibition[a]	K_i[b] (μM)	Human specific activity (nmol/mg min)	Inhibition[a]
1	9-[β-D-ribofuranosyl]-adenine (adenosine)	4000 ± 450			2400 ± 750	
N^1-position						
2	Adenosine-N^1-oxide	15 ± 0.4	+		2800 ± 420	
3	N^1-Methyl-adenosine	21 ± 2	+++			+
4	N^1-Ethyl-adenosine	<1	+			+
5	N^1-Benzyl-adenosine	<0.5	+++	0.19 c		+
6	1-(4-Fluorobenzyloxy)-adenosine	<3	+			
7	N^1-Oxy-N^6-methyl-adenosine	15	+		1700 ± 220	
8	N^1-Oxy-2'-deoxy-adenosine	<2	–			
2-position						
9	2-Aza-adenosine	4030 ± 470	++		1800 ± 400	
10	2-Fluoro-adenosine	2070 ± 430	+++	0.5 c	1800 ± 220	+
11	2-Chloro-adenosine	460 ± 60	++		16 ± 3	
12	2-Bromo-adenosine	13 ± 2	++		8 ± 1	
13	2-Amino-adenosine	240 ± 15	+		120 ± 30	
14	2-Azido-adenosine	4 ± 1	++			
15	2-Hydroxy-adenosine	6 ± 1	+			
16	2-Methyl-adenosine	74 ± 2	+		4 ± 0.6	
17	2-Trifluoromethyl-adenosine	<1	+			
18	2-Methoxy-adenosine	2 ± 0.4	++			
19	2-Ethyl-adenosine	<1	+			
20	2-(1-Ethyn-1-yl)-adenosine	<1	+			
3-position						
21	3-Deaza-adenosine	1.4 ± 0.3	+		<1	
22	2-Fluoro-3-deaza-adenosine	68 ± 14	+		<1	
6-position						
23	N^6-Amino-adenosine	<2	+			
24	9-[β-D-Ribofuranosyl]-purine (purine riboside)	66 ± 11	+		3300 ± 1300	
25	6-Chloro-purine riboside	110 ± 13	++		2500 ± 1200	
26	6-Bromo-purine riboside	87 ± 14	++		2500 ± 390	
27	6-Iodo-purine riboside	<3	+			
28	6-Oxy-purine riboside (inosine)	<3	–			
29	6-Mercapto-purine riboside	<4	+			
30	6-Methyl-purine riboside	110 ± 6	++		980 ± 100	
31	6-Fluoromethyl-purine riboside	85 ± 6	++		2400 ± 720	
32	6-Methoxy-purine riboside	3 ± 1	+++			+
33	6-Methylmercapto-purine riboside	<1	+++			+
34	6-Cyclopentyloxy-purine riboside	<2	+++	0.15 m		+
35	6-Nitrobenzyl-mercapto-purine riboside	<1	+++			+
36	6-Benzyl-mercapto-purine riboside	<1	+++			+
2- and 6-positions						
37	2,N^6-Dimethyl-adenosine	2.4 ± 0.6	+			
38	2-Methyl-N^6-octyl-adenosine	Solubility[c]	++			
39	2-Methyl-N^6-benzoyl-adenosine	<0.2	+			
40	2-Chloro-purine riboside	1 ± 0.2	+			
41	2-Amino-6-oxypurine riboside (guanosine)	<1	+			
42	2-Methyl-6-oxypurine riboside	<2	+			
43	2-Chloro-6-methoxy-purine riboside	2.0 ± 0.3	++			
44	2,O^6-Dimethyl-inosine	1.2 ± 0.4	++			

Table 1 (*Continued*)

	Compound name	*M. tuberculosis* specific activity (nmol/mg min)	Inhibition[a]	K_i[b] (μM)	Human specific activity (nmol/mg min)	Inhibition[a]
45	2-Azido-O^6-methyl-inosine	1 ± 0.06	+++			+
46	2-Methyl-S^6-phenyl-6-mercaptopurine riboside	<2	+			
7-position						
47	7-Deaza-adenosine (tubercidin)	<2	++			
48	7-Iodo-7-deaza-adenosine (iodotubercidin)	<2	+++	0.21 c [10]		++++
49	7-Cyano-7-deaza-adenosine (toyocamycin)	0.7 ± 0.2	+++			+++
50	7-Deaza-7-carboxamido-adenosine (sangivamycin)	<2	++			
8-position						
51	8-Aza-adenosine	160 ± 50	++		910 ± 400	
52	8-Azido-adenosine	0.8 ± 0.06	+			
53	8-Chloro-adenosine	0.2 ± 0.06	+			
54	8-Bromo-adenosine	<1	+			
55	8-Aza-9-deaza-adenosine (formycin A)	4422 ± 380	++	5.8 m	600 ± 86	+
56	2-Fluoro-8-aza-adenosine	150 ± 11	++		220 ± 12	
6- and 8-positions						
57	6-Fluoro-8-amino-purine riboside	<1	+			
58	8-Chloro-purine riboside	<1	+			
59	8-Bromo-purine riboside	<4	+			
60	8-Hydroxy-purine riboside	<3	+			
61	8-Methoxy-purine riboside	<3	+			
62	8-Amino-purine riboside	<2	+			
63	8-Dimethylanimo-purine riboside	<2	+			
64	8-Mercapto-purine riboside	<1	+			
65	8-Methylmercapto-purine riboside	<1	–			
66	8-Azido-purine riboside	<1	+			
9-position						
67	9-Deaza-adenosine	<1	++			
Multiple substitutions						
68	1-Deaza-2-amino-6-chloro-purine riboside	<2	+			
Other compounds						
69	Cytidine	<2	+			
70	Thymidine	<2	–			
71	Uridine	<2	+			
72	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin)	3 ± 0.6	–			
73	2-β-D-Ribofuranosylthiazole-4-carboxamide (Tiazofurin)	<1	–			
74	2-β-D-Ribofuranosylselenazole-4-carboxamide (Selenazofurin)	<1	–			
75	2′-Deoxycoformycin (pentostatin)	<2	+			
76	6-dimethylamino-9-[3-deoxy-3-(*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]-β-purine (puromycin)	<1	–			
77	9-(*Cis*-2-cyclopentyl)-purine	<1	–			
78	1-β-D-Ribofuranosyl-imidazo-[1,2-β]-pyrazole-7-carbonitrile	<1	–			
79	N-[4-(Benzyloxy)amidino]-1-β-D-ribofuranosyl imidazo-9-yl-formamide	<1	++			
80	4-Carboxamide-5-amino-1-β-D-arabinofuranosyl-imidazole	<2	–			
81	3-Amino-1-β-D-ribofuranosyl-triazolo-[5,1-c]-*S*-triazole	<1	+			

[a] Inhibition of 0.1 μM adenosine phosphorylation is indicated as follows: '–', <10% inhibition at 100 μM of compound; '+', 10–90% inhibition at 100 μM; '++', 10–90% inhibition at 10 μM; '+++', 10–90% inhibition at 1 μM; '++++', 10–90% inhibition at 0.1 μM.

[b] The manner of inhibition is denoted by a 'c' or 'm' for competitive or mixed inhibition, respectively.

[c] During the processing of samples for HPLC, this compound precipitated out of solution rendering it impossible to be evaluated by this method.

(**73**), 2-β-D-ribofuranosylselenazole-4-carboxamide (**74**) and 2′-deoxycoformycin (**75**). The National Cancer Institute (Bethesda, MD) provided 7-deaza-7-carboxamido-Ado (**50**). 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (**72**) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). [2,8-^{3}H]-Ado used in the inhibition studies was purchased from Moravek Biochemicals (Brea, CA). All other compounds were provided by the chemical repository at Southern Research Institute (Birmingham, AL). Each compound was solubilized in water or DMSO as necessary.

2.2. *M. tuberculosis* Ado kinase

M. smegmatis strain SRI101-pVV16/*adoK*, which contains the *M. tuberculosis* Ado kinase gene cloned into an Ado kinase-deficient strain of *M. smegmatis*, was used as a source of *M. tuberculosis* Ado kinase [10]. Protein extracts were prepared and Ado kinase was purified as previously described [10]. Protein concentrations were determined at each step by the Bradford method using bovine serum albumin (BSA) as a standard [15].

2.3. Human Ado kinase

The human Ado kinase clone 20-1 was generously provided by Dr. Jozef Spychala (UNC Chapel Hill, Chapel Hill, NC). *Escherichia coli* strain BL21 was transformed with a pET24b vector containing the human Ado kinase gene [11]. Bacteria were grown at 37 °C in LB media supplemented with 50 μg/ml kanamycin. In order to induce protein expression, clone 20-1 was grown at 22 °C to an OD of 0.6. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and expression was allowed to proceed for 4 h. Cells were pelleted by centrifugation, rinsed once with an equal volume of phosphate buffered saline, then rinsed twice with 50 mM HEPES (pH 6) containing 100 mM KCl, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride, and Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and resuspended in the same buffer. Resuspended *E. coli* BL21 were disrupted by sonication, and crude protein extracts were prepared as described for *M. tuberculosis* Ado kinase [10].

Crude protein extracts were subjected to precipitation with 70% ammonium sulfate and dialyzed against 50 mM HEPES (pH 6.0) containing 100 mM KCl, 1 mM DTT, and 20% glycerol (buffer A). Ado kinase activity was found in the >70% ammonium sulfate fraction. Human Ado kinase was then applied to a 5′-AMP-Sepharose-4B affinity column, which was washed in a stepwise manner with 20 ml of buffer A followed by 10 ml of buffer A containing 1 M KCl, 10 ml of buffer A containing 20 μM Ado, 10 ml of buffer A with 20 μM ATP, and a final rinse with 15 ml of buffer A. Human Ado kinase eluted with the rinse containing buffer A with 20 μM Ado. The purified protein preparation was found to be free of AMP kinase, but some Ado deaminase activity remained, therefore deoxycoformycin was included in all assays involving the human enzyme.

2.4. Substrate assays

Nucleosides were assayed as substrates for *M. tuberculosis* Ado kinase, and reaction products were detected by HPLC. Assay conditions consisted of 50 mM Tris–HCl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 0.01% BSA, 10 μM deoxycoformycin, and 100 μM of the appropriate test compound. Human Ado kinase was assayed similarly with the following changes: assay conditions consisted of 50 mM HEPES (pH 6.0), 40 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1% BSA, 10 μM deoxycoformycin, and 100 μM of the appropriate test compound. Reactions were started by the addition of enzyme and incubated in a 37 °C water bath. Aliquots of 50 μl were taken at 0, 20, 40, and 60-min intervals and reactions were stopped at each timepoint by the addition of 50 μl of 1 M perchloric acid. Samples were neutralized to pH 7 and precipitated salts were removed by centrifugation. Reactants and products were separated by HPLC using Bio Basic anion exchange column (Thermo Electron Corp., Bellefonte, PA) with a 30-min linear salt and pH gradient from 6 mM ammonium phosphate (pH 2.8) to 900 mM ammonium phosphate (pH 6). Peaks were detected as they eluted from the column by absorbance at their λ_{max}, typically between 260 and 320 nm. All enzyme reactions were linear during the incubation period and substrate conversions were maintained at less than 10%.

2.5. Inhibition assays

Assay mixtures were identical to those performed for HPLC analysis with the addition of 0.1 μM [^{3}H] Ado (4 μCi/ml) and 100 μM of the test compound. Reactions were started by the addition of enzyme, incubated for one hour at 37 °C, and stopped by the addition of 10 μl of 0.1 M EDTA. At appropriate timepoints, 50 μl aliquots were applied to a DE-81 cellulose disk and allowed to dry. Disks were batch-washed three times with 1 mM ammonium acetate (pH 5.0), rinsed with 95% ethanol, and dried. Filter disks were transferred to scintillation vials with 10 ml of Complete Counting Cocktail (Research Products International, Mount Prospect, Illinois), and radioactivity was detected with a Packard Tri-Carb model 1900 TR liquid scintillation analyzer. Enzymatic activity was calculated from the amount of radioactivity that bound to the DE-81 disks. Compounds were ranked by their ability to inhibit the phosphorylation of 0.1 μM Ado, and compounds that inhibited by 90% or greater were re-assessed at 10 μM. This iterative process was continued with serial 10-fold dilutions of a test compound until the compound no longer inhibited Ado phosphorylation by ≥90%.

Inhibition constants were determined by assaying various concentrations of inhibitor in the presence of increasing concentrations of Ado. Double-reciprocal plots were created and replots of the slopes of the double-reciprocal plots versus concentration of inhibitor were used to determine the K_i. Sigma plot version 8.02 enzyme kinetics module version 1.1.1 was used to analyze the data.

2.6. Determination of minimum inhibitory concentration (MIC)

MIC values were evaluated in *M. tuberculosis* strains H37Ra, an Ado kinase-deficient strain derived from *M. tuberculosis* H37Ra (SRICK1), and SRICK1 complemented with the *adoK* gene (SRICK1::*adoK*), using a colorimetric microdilution broth assay as previously described [10].

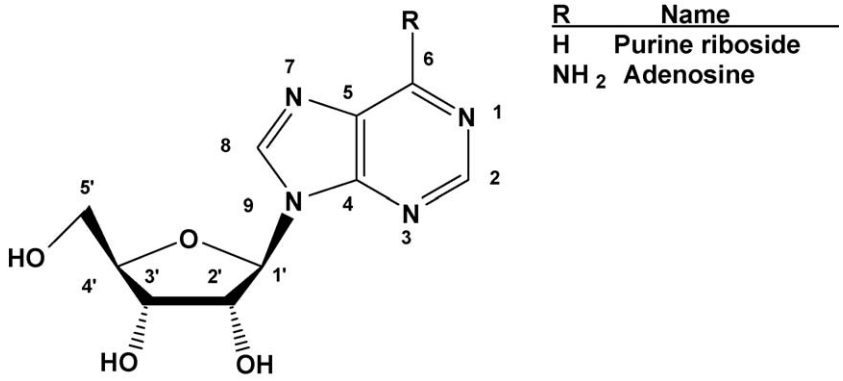

Fig. 2 – Structure and numbering convention for purine riboside and Ado.

3. Results

The purpose of this structure–activity relationship (SAR) was to utilize a structurally diverse set of ribonucleosides to probe the active site of *M. tuberculosis* Ado kinase in order to discern the requirements for both Ado kinase substrates and inhibitors. Toward this end, 80 nucleoside analogs were evaluated containing either endocyclic substitutions, exocyclic substitutions, or a combination of both on the adenine ring of Ado (Table 1). Fig. 2 contains the numbering convention used in naming these compounds. Most of these ribonucleosides were modified Ado analogs since previous studies demonstrated that Ado was the best substrate of the natural nucleosides [10]. In this study, substrate activity was measured directly using HPLC to detect product formation without the need of radiolabeled substrates. This method provided excellent resolution of the monophosphate peaks from the parent nucleoside (Fig. 3), and permitted calculations of specific activity.

Since this SAR was a part of the drug development process, knowledge of the substrate and inhibitor specificity of Ado kinase from the human host was also desirable. Therefore, compounds that were either good substrates or inhibitors were further tested with human Ado kinase. Many of the compounds that we tested against human Ado kinase had been previously studied with human, rabbit liver, and *Toxoplasma gondii* Ado kinases, however the methods employed in those studies varied and therefore were not quantitatively comparable [16–19]. The value of testing these compounds with human Ado kinase again is to permit a quantitative comparison of results with those found in the *M. tuberculosis* homolog. Results are summarized in Table 2.

3.1. *Modification of the purine base*

Sixty-seven compounds were tested with single or multiple modifications to the base moiety of Ado or purine riboside.

3.1.1. N^1 *position*

Compounds with substitutions to the N^1 position resulted in at least a 99% decrease in activity relative to Ado in *M. tuberculosis* Ado kinase, whereas they are excellent substrates for human Ado kinase. In the *M. tuberculosis* enzyme, the best substrate was Ado-N^1-oxide (**2**) with 0.3% of the activity of Ado (**1**). However, no aromatic substitutions at the N^1 position were tolerated as substrates. These results suggest that small size was the main predictor of substrate activity for this site.

Unlike *M. tuberculosis* Ado kinase, substitutions to the N^1-position of human Ado kinase resulted in highly active compounds. Ado-N^1-oxide (**2**) was as good a substrate as Ado (**1**). However, the double-substitution N^1-oxy-N^6-methyl-Ado (**7**) was 40% less active than Ado-N^1-oxide (**2**). These results were in agreement with previous studies [19].

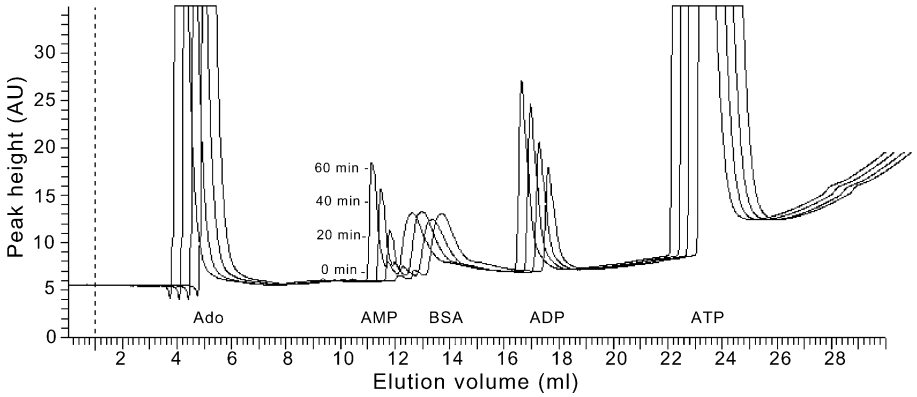

Fig. 3 – BioBasic anion exchange HPLC of Ado and its phosphorylated products. The overlay of this typical timecourse demonstrates the phosphorylation of Ado by *M. tuberculosis* Ado kinase at 0, 20, 40, and 60 min. Timepoints are offset by a 3° rotation.

All of the compounds tested as inhibitors of human Ado kinase were poor inhibitors, only moderately active at 100 μM, whereas the N^1-position proved promising for inhibition of the *M. tuberculosis* enzyme. Indeed, N^1-benzyl-Ado (**5**) was a potent competitive inhibitor with a K_i of 0.19 ± 0.1 μM.

3.1.2. 2-position

An endocyclic substitution at the 2-position, 2-aza-Ado (**9**), conserved 100% of the activity observed with Ado. The main predictor for activity in exocyclic substitutions at the 2-position was small size. The relative activities of fluorine, chlorine, and bromine substitutions at the 2-positions (**10**, **11**, and **12**, respectively) illustrated the limitations of size at this position with respective activities of 2070, 460, and 13 nmol/mg min. Within substitutions of approximately the same size, the second predictor for activity was electronegativity of the compound, with electropositive substitutions preferred over electronegative ones. Pairs of similarly sized substitutions illustrated this best; hydrogen (**1**) was preferred over fluorine (**10**) by two-fold ($p < 0.01$), and a methyl-group (**16**) was preferred over trifluoromethyl (**17**) by greater than 60-fold ($p < 0.01$). Methyl-Ado (**16**) was a poor substrate with 74 nmol/mg min of activity (2% of the activity of Ado).

Substitutions at the 2-position followed a similar pattern for human Ado kinase, with size and electronegativity as the main determinants of substrate activity. However, substitutions at this position did not maintain the same level of activity that was seen in the *M. tuberculosis* homolog. 2-Aza-Ado (**9**) was an excellent substrate for human Ado kinase, maintaining 75% of the activity seen with Ado. This compound and its analogs have been extensively studied as substrates for human Ado kinase and our results were in agreement with previous reports [17,19]. Whereas 2-aza-Ado (**9**) and 2-fluoro-Ado (**10**) were the best substrates in this category, activity was dramatically decreased with increasing size of the exocyclic substitution. The series including fluoro, chloro, and bromo (**10**, **11**, and **12**) substitutions at the 2-position illustrate this well, respectively maintaining 76%, 0.7%, and 0.3% of the activity of Ado. These results agree with previous reports which included a more extensive evaluation of exocyclic substitutions at this site [17,19–22].

Differences in the 2-position of the active sites of these two Ado kinases are illustrated by inhibition of Ado phosphorylation by 2-fluoro-Ado (**10**). Among the 2-substituted Ado analogs, the most potent inhibitor of *M. tuberculosis* Ado kinase was 2-fluoro-Ado (**10**) with a K_i of 0.5 ± 0.1 μM and a competitive mode of inhibition. However, it only inhibited human Ado kinase at 100 μM, the highest concentration tested. This disparity indicated that 2-fluoro-Ado (**10**) had a much lower K_m with *M. tuberculosis* Ado kinase than human.

3.1.3. N^3-position

The endocyclic substitution of a carbon at the 3-position, 3-deaza-Ado (**21**), eliminated >99.9% of the activity of the enzyme, suggesting that the N^3-endocyclic nitrogen may be important for substrate recognition, potentially functioning as a hydrogen bond acceptor. This loss of activity may be partially overcome with a second substitution as demonstrated by 2-fluoro-3-deaza-Ado (**22**) which was more active than 3-deaza-Ado (**21**) and maintained selectivity for *M. tuberculosis* Ado kinase. Consistent with previous reports, neither of these compounds were substrates for human Ado kinase at detectable levels [17,23].

3.1.4. 6-position

Human and *M. tuberculosis* Ado kinases had large differences in the substrate specificity for 6-substituted purine ribonucleosides. All of the compounds tested with human Ado kinase (**24–26**, **30**, and **31**) were excellent substrates with as much or more activity than Ado with the exception of 6-methyl-purine riboside (**30**) which was 41% as active as Ado. That 6-substituted ribonucleosides are excellent substrates is in qualitative accordance with previous studies of human and mammalian Ado kinases, however the results vary quantitatively from previous reports [17,19]. These compounds were reported to be several-fold better substrates than Ado in human Ado kinase, whereas they were relatively poor substrates for rabbit liver Ado kinase, a prototypical mammalian form [17,19]. This disparity may reflect differences in conditions for assaying these compounds.

In *M. tuberculosis* Ado kinase, N^6-amino-Ado (**23**) demonstrated a >99% decrease in activity. Purine riboside (**24**) and its 6-substituted analogs (**25–36**) were poor substrates, however several were at least 10-fold better as substrates than N^6-amino-Ado. Exocyclic substitutions at the 6-position of purine riboside up to the size of a methyl group were substrates if they took the lactim (enol) form, such as 6-chloro-purine riboside (**25**) and 6-bromo-purine riboside (**26**). However, compounds of similar size and electronegativity such as 6-oxy-purine riboside (**28**) or 6-mercapto-purine riboside (**29**) were not substrates since the modification resembled the lactam (keto) tautomeric form. These results were similar to observations made with *T. gondii* Ado kinase [16]. Of the 6-position substitutions made to purine riboside (**24**, 66 nmol/mg-min), 6-chloro, -bromo, and -methyl (**25**, **26** and **30**) groups were at least as active as purine riboside (**24**) with specific activities of 110, 110, and 87 nmol/mg min, respectively. The largest substitution that still maintained substrate activity was a 6-methoxy group (**32**). N^6-amino-adenosine (**23**) was similar in size to 6-methoxy-purine riboside (**32**) but was not a substrate, indicating that size is not the only factor that is important for substrate activity at this site.

Several 6-substituted analogs were excellent inhibitors of *M. tuberculosis* Ado kinase but not human. These inhibitors fell into one of two categories. The first category included compounds in which the substitution was at least three-atoms long with a proximal electronegative component such as a sulfur, oxygen, or nitrogen, combined with a distal methyl group. Similarly, the second group had a proximal electronegative component such as an oxygen or sulfur combined with a large, cyclic component such as a cyclopentyl or benzyl substituent. One of the most potent inhibitors, 6-cyclopentyloxy-purine riboside (**34**), demonstrated a mixed mode of inhibition and a K_i of 0.15 ± 0.08 μM.

3.1.5. 7-position

7-Deaza-Ado (**47**) and its analogs are excellent substrates for human, rabbit, and *T. gondii* Ado kinases and are known as excellent inhibitors as well [17,24–28]. This series of compounds also proved to be excellent inhibitors of *M. tuberculosis*

Ado kinase, with 7-iodo-7-deaza-Ado (**48**) and 7-cyano-7-deaza-Ado (**49**) among the best inhibitors in this class. No 7-deaza-Ado analogs were effective substrates for *M. tuberculosis* Ado kinase. Although they were excellent inhibitors of *M. tuberculosis* Ado kinase, the inability of the 7-deaza series to act as substrates for this enzyme highlights the importance of N^7 for substrate activity.

3.1.6. 8-position

Consistent with previous reports, 8-aza-Ado (**51**), was a good substrate for human Ado kinase, maintaining 38% of the activity of Ado [17,19,20]. This compound was also the best substrate of the 8-substituted analogs tested against *M. tuberculosis* Ado kinase, with a specific activity of 160 nmol/mg min, or 4% of the activity seen with Ado. Exocyclic substitutions were significantly worse substrates than 8-aza-Ado. Addition of a 2-fluoro group, 2-fluoro-8-aza-Ado (**56**), was as active as 8-aza-Ado (**51**) and improved the selectivity for *M. tuberculosis* Ado kinase relative to 8-aza-Ado (**51**). Likewise, exocyclic substitutions to the 8-position proved to be poor inhibitors, whereas the addition of an endocyclic N, 8-aza-ado (**51**) and 2-fluoro-8-aza-Ado (**56**), proved to be good inhibitors.

3.1.7. 9-position

The endocyclic substitution at the 9-position, 9-deaza-Ado (**67**), had a specific activity of < 1 nmol/mg min suggesting that the endocyclic nitrogen is important for substrate activity, possibly acting as a hydrogen bond acceptor. However, 9-deaza-Ado (**67**) proved to be a good inhibitor.

3.1.8. 8- and 9-positions

Although it could not have been predicted based on the activities of each individual substitution, 8-aza-9-deaza-Ado (formycin A, **55**) proved to be one of the best substrates for *M. tuberculosis* Ado kinase of all of the compounds tested, with a specific activity of 4400 nmol/mg min (110% of the activity of Ado). This compound was also a substrate for human Ado kinase, with 25% of the activity of Ado for this enzyme. This activity conflicted with a previous report that indicated that it was at best a poor substrate for mammalian Ado kinase [17]. Formycin A (**55**) also proved to be a good inhibitor of *M. tuberculosis* Ado kinase with a mixed mode of inhibition and a K_i of 5.8 ± 2.1 μM.

3.2. Alternative base structures

Of the 13 ribosides tested that had a pyrimidine-base or alternative ring structures, most were not substrates for Ado kinase with specific activities <2 nmol/mg min. One exception to this was 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin, **72**), which had a specific activity of 3 nmol/mg min. This nucleoside analog is a known substrate for human Ado kinase and is used clinically as an effective antiviral agent [29]. Ribavirin resembles N^1-substituted Ado, but with an open ring between N^1 and C^2. The only compound in this category that inhibited Ado kinase was *N*-[4-(benzyloxy) amidino]-1-β-D-ribofuranosyl imidazo-9-yl-formamide (**79**).

3.2.1. MIC assays

Several nucleosides were selected for MIC analysis with *M. tuberculosis* H37Ra, SRICK1, and SRICK1::*adoK* (Table 3). Compounds were selected based on their unique structures or substrate activity, which ranged from 74 to 4030 nmol/mg min. Of the compounds tested, several 2-substituted Ado analogs were promising in terms of antimycobacterial activity. The specific activity of the compound as measured by our assays

Table 2 – Summary of results

Position	Result
N^1	Substrate activity is associated with small size of exocyclic substitutions. Compounds in this group may have limited utility because they were better substrates for human than *M. tuberculosis* Ado kinase. Inhibition is associated with large substitutions including aromatic or aliphatic groups. This position was among the most promising for the design of selective Ado kinase inhibitors
C^2	Substrate activity is preserved by the substitution of C^2 with an endocyclic N. Exocyclic substitutions should be small in size, with electropositive substitutions preferred over electronegative ones for substitutions of similar size. The best inhibitors at this position had small, electronegative exocyclic substitutions
N^3	The endocyclic N was very important for substrate activity, perhaps acting as a hydrogen bond acceptor. Loss of activity with 3-deaza-Ado (**21**) may be partially overcome by the addition of an additional favorable substitution as demonstrated by 2-fluoro-3-deaza-Ado (**22**). Compounds should be further investigated in the 3-deaza-Ado series because they were promising for selectivity
C^6	An exocyclic amino group was preferred at the 6-position. Other 6-substituted purine ribosides maintained no more than 3% of the activity of Ado. Small, electropositive substitutions may be substrates in the lactim (enol) tautomeric form. All of the 6-substituted compounds tested were better substrates for human Ado kinase than *M. tuberculosis* Ado kinase, however the opposite was true for inhibitors. 6-Substituted purine ribosides were much better inhibitors of *M. tuberculosis* Ado kinase than human. Inhibitors fell into two categories, the first had a proximal electronegative atom (O, N, S) with a distal methyl group. Similarly, the second group had a proximal electronegative atom with a distal cyclic component such as cyclopentene or phenyl group
C^7	An endocyclic nitrogen was essential for substrate activity, however compounds in the 7-deaza-Ado series were potent inhibitors of *M. tuberculosis* and human Ado kinases
C^8	An endocyclic nitrogen was the best substrate and inhibitor of the 8-substituted Ado analogs. Substrate activity and inhibition diminished with the addition of an exocyclic substitution, perhaps due to steric hindrance
N^9	Replacement of N^9 with an endocyclic carbon resulted in abolition of activity. However, 8-aza-9-deaza-Ado (**55**) was one of the best substrates and a potent inhibitor of *M. tuberculosis* Ado kinase. It is likely that N^8 can act as a critical H-bond acceptor compensating for the loss of N^9

Table 3 – MIC results with selected nucleosides

Compound name	Specific activity (nmol/mg min)	MIC[a] (μg/ml)		
		H37Ra	SRICK1	SRICK1::*adoK*
2-Aza-Ado	4030	1–10	10–100	1–10
2-Fluoro-Ado	2070	1–10	1–10	0.1–1
2-Chloro-Ado	460	1–10	10–100	1–10
2-Amino-Ado	240	>100	>100	>100
2-Methyl-Ado	74	1–10	>100	0.1–1
8-Aza-9-deaza-Ado	3808	>100	>100	>100

[a] MIC assays were performed at least twice for each compound.

was not predictive of efficacy against intact bacteria. Indeed, 8-aza-9-deaza-Ado was less effective against the intact organism than 2-methyl-Ado even though 8-aza-9-deaza-Ado was a much better substrate for Ado kinase. Lack of antibacterial activity for compounds that are good substrates could be caused by reduced transport, increased efflux, metabolism through an alternative enzyme such as Ado deaminase, or failure to inhibit a downstream target.

This assay was useful to begin to delineate the mechanism of action for Ado analogs that demonstrated activity. Loss of activity in SRICK1, as in the case of 2-methyl-Ado, indicated that the compound worked predominantly through Ado kinase. If the MIC in SRICK1 was greater than in H37Ra, but still measurable as in the case of 2-aza-Ado, 2-fluoro-Ado, and 2-chloro-Ado, then the mechanism of action is likely to involve Ado kinase as well as an alternative pathway.

4. Discussion

M. tuberculosis Ado kinase is the only Ado kinase to be identified and purified from a bacterium. Initial characterization of the enzyme revealed that it had biochemical and physical properties that differed from other known Ado kinases [10,12]. Therefore, this enzyme may be utilized for drug development by providing a selective pathway for the conversion of nucleoside analogs to biologically active metabolites as in the case of methyl-Ado (**16**) [7–9]. The goal of this study was to identify structural modifications to the adenine moiety of Ado that would result in phosphorylated products. This would provide a better understanding of the topography of the active site and aid in the design of other analogs that could be selectively phosphorylated by *M. tuberculosis* Ado kinase. To this end, compounds were assayed as both substrates and inhibitors, and the results provided a comprehensive picture of the active site of *M. tuberculosis* Ado kinase that will be useful for rational drug development.

Since the compounds that we tested were analogs of the natural substrate it would have been more economical in terms of time and money to have tested the compounds as inhibitors only, and inferred that the best inhibitors would also be the best substrates. However, we found that there was little correlation between the ability of a compound to inhibit Ado phosphorylation and its ability to be a substrate. There are several reasons for this. Substrate activity is a reflection of the affinity of a compound for the active site and the reaction rate. These parameters will vary from one compound to another. Furthermore, there are different mechanisms of inhibition including competitive, non-competitive, uncompetitive, and mixed-type. If the compound is any other than a competitive inhibitor, it is unlikely to be a substrate for the enzyme. Since substrate and inhibitor activity are dependent on both the affinity for the active site and the reaction rate, caution should be taken when interpreting inhibition as a surrogate for substrate activity.

While it would be useful to have K_i, K_m and V_{max} values for these compounds, it would be impractical to perform the necessary studies for all of the compounds that we tested. The measurement of inhibition that we report can be considered a crude estimate of the I_{50} (amount of compound required to inhibit enzymatic activity by 50%) for the enzyme under these conditions where the margin of error is no more than five-fold. For competitive inhibitors, the relative affinity of each compound may be determined by applying the relationship of Cheng and Prusoff [30] where $(I_{50})_1/(I_{50})_2 = (K_i)_1/(K_i)_2$. This relationship holds true only if the concentration of substrate is constant (0.1 μM in this work), since the I_{50} is dependent on [S]. The compounds for which the K_i value was determined give us a guideline for the affinity of compounds that inhibited phosphorylation of 0.1 μM Ado at a level of 1 μM. Most of these compounds had K_i values in the 100 nM range. Therefore, compounds that were inhibitors at 10 μM had a 10-fold weaker affinity for the active site than compounds that inhibited at 1 μM, and their K_i values should be in the low μM range.

Similarly, for compounds that had both substrate and inhibitor activity, the specific activity measured at 100 μM is likely to be a good estimate of V_{max} since the measurement was made at a concentration that is likely greater than the K_m for these compounds. As in the case of 2-fluoro-Ado (**10**), this estimation of V_{max} is more precise for compounds that inhibited at 10 or 1 μM (i.e. had greater affinity and lower K_m) than for compounds that inhibited only at 100 μM. In this manner, we can begin to get an idea for the Michaelis–Menton parameters for these compounds. Furthermore, some of the most potent inhibitors were not substrates at all, such as the 7-deaza-Ado series (**47–50**) and some 6-substituted purine ribosides (**32–36**). Results such as this indicate that these compounds would have a low K_m and low V_{max}. Review of the results in this manner can provide an estimate of the Michaelis–Menton parameters for these compounds.

Three of the five inhibitors that were selected for K_i studies were competitive with Ado and two were mixed inhibitors. For

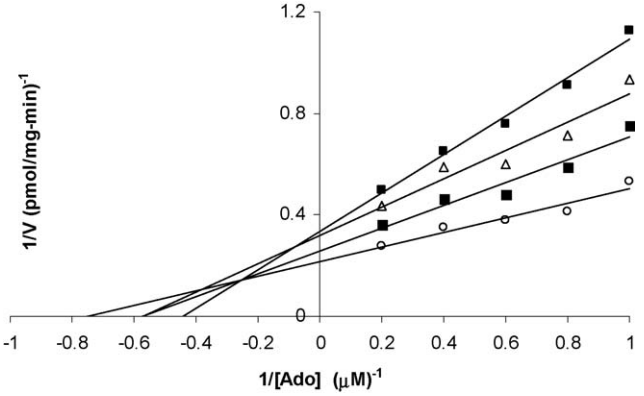

Fig. 4 – Lineweaver–Burke plot of regressed data for 8-aza-9-deaza-Ado. The data presented in this figure are the results of a single experiment. The experiment was performed three times with similar results. The quality of the fit for each regression with different concentrations of 8-aza-9-deaza-Ado are as follows: (○) 0 μM, R = 0.97; (■) 4 μM, R = 0.97; (△) 8 μM, R = 0.96; (◆) 12 μM, R = 0.99.

the mixed inhibitors, the intersection of the double-reciprocal plots occurred in the quadrant to the left of the 1/V axis and above the 1/[S] axis (Fig. 4). The decision to call these inhibitors mixed-type instead of competitive was based on a judgment that this intersection was real, although it was very close to the 1/V axis. The judgment call was affirmed by the SigmaPlot analysis, which also determined a mixed mode of inhibition for these compounds. Mixed-type inhibition can be the result of competitive and uncompetitive inhibition, which is likely in this case. Since ATP contains Ado, the active site for ATP may also bind Ado analogs. Indeed, high resolution crystal structures of Ado kinase from both human and *Toxoplasma gondii* sources have revealed an Ado molecule bound to the ATP-binding site [31,32]. Since crystal structures for these enzymes did not reveal an allosteric site for Ado, it is possible that this phenomenon also accounts for substrate inhibition that has been previously reported for Ado kinases from human and *M. tuberculosis*, sources [10,33].

Drug development efforts are focused on using Ado kinase as a filter for the selective activation of competing alternative substrates in *M. tuberculosis*, not on inhibiting this enzyme. Therefore, it is easy to place emphasis on compounds with greater specific activity as a measure of the potency of the compound. Good substrates will produce more of the phosphorylated product and increase the likelihood of inhibiting a downstream target. However, 2-methyl-Ado (16) demonstrated that even a poor substrate can be an effective antitubercular compound. As long as the phosphorylated product is potent enough to preferentially inhibit its downstream target, very little of it may be needed in order to have a desirable effect. For this reason, we have focused on substitutions that may be made to the Ade moiety of Ado that will result in selectively phosphorylated products. Comparison of the results for *M. tuberculosis* and human Ado kinases has provided an invaluable tool for drug development because it highlighted modifications that would improve the selectivity for *M. tuberculosis* Ado kinase.

Current drug development efforts revolve around the design of subversive substrates, however it is possible that development of specific Ado kinase inhibitors will be beneficial in the future. Ado kinase-deficient strains of *M. tuberculosis* survive well in vitro, however nothing is known about the impact that Ado kinase-deficiency will have on the growth and survival of the organism in vivo. It is uncommon for a bacterium to have Ado kinase activity, and it is possible that *Mycobacterium* spp. have this function in order to survive in macrophage during infection. Until more is known about the physiological impact of Ado kinase inhibition, compounds identified as good inhibitors serve to provide information about the active site and are not considered as lead compounds for drug development.

Inclusion of inhibition studies in this work provided a more complete picture of the Ado binding domain than would be achieved by substrate studies alone. Inhibition studies revealed the presence of hydrophobic pockets at the N^1 and 6-positions similar to those seen with human and *T. gondii* Ado kinases [16,18,19,31,34]. It is possible that the hydrophobic pockets at the N^1 and 6-positions are actually a single, large pocket that encompasses the whole area. Unfortunately, this work is unable to clarify the nature of the hydrophobic pocket(s), thus they will be considered separately for the sake of discussion. At both of these sites, interaction of a compound with the hydrophobic pocket increased the likelihood that a compound would be an inhibitor and not a substrate. Furthermore, the poor substrate and inhibitor activities of compounds with exocyclic substitutions at the 8-position suggests that steric hindrance may come into play at this site. These types of results permit the formulation of a model for the topography of the Ado binding domain (Fig. 5) in the

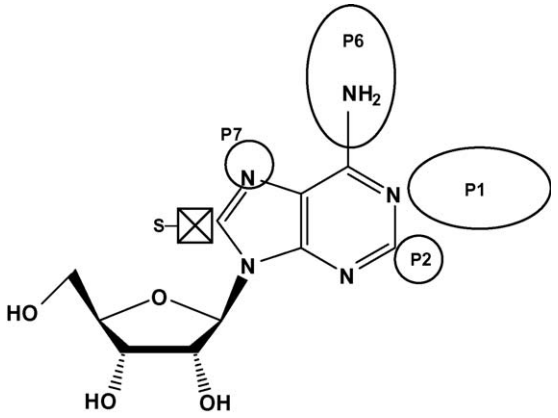

Fig. 5 – Schematic representation of structural features of the Ado-binding domain. P1 and P6 are the hydrophobic pockets found at $N^1$and C^6, respectively. P6 is at least large enough to accommodate a nitrobenzyl-mercapto-moiety. P1 is at least large enough to accommodate a *p*-fluorobenzyloxy moiety. Interaction with these two pockets is predictive for inhibition. P2 is a pocket at the 2-position that is at most large enough to accommodate a methoxy group, interaction at this site is predictive for substrate activity. A pocket also exists at the 7-position that is at least large enough to bind a carboxamido group. The 'S' at the 8-position denotes a steric blockade for exocyclic substitutions at this site.

absence of a high resolution crystal structure and provide a guide for the design of more Ado analogs.

This SAR permitted development of a model for the Ado-binding domain of Ado kinase and highlighted a few sites on the adenine base that may be useful for development of nucleoside analog antitubercular compounds. This study focused on the issue of selectivity at the level of Ado kinase by the inclusion of human Ado kinase studies, however further studies must be done to determine efficacy and cytotoxicity of these compounds. This comparison between human and *M. tuberculosis* Ado kinases has highlighted several differences in the active sites of these enzymes that may be exploited for rational drug development.

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