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Structure-activity relationship for adenosine kinase from

Mycobacterium tuberculosis:

II. Modifications to the ribofuranosyl moiety

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Abstract

Adenosine kinase (Ado kinase) from *Mycobacterium tuberculosis* is structurally and biochemically unique from other known Ado kinases. This purine salvage enzyme catalyzes the first step in the conversion of the adenosine analog, 2-methyl-Ado (methyl-Ado), into a metabolite with antitubercular activity. Methyl-Ado has provided proof of concept that the purine salvage pathway from *M. tuberculosis* may be utilized for the development of antitubercular compounds with novel mechanisms of action. In order to utilize this enzyme, it is necessary to understand the topography of the active site to rationally design compounds that are more potent and selective substrates for Ado kinase. A previous structure-activity relationship identified modifications to the base moiety of adenosine (Ado) that result in substrate and inhibitor activity. In an extension of that work, sixty-two Ado analogs with modifications to the ribofuranosyl moiety, modifications to the base and ribofuranosyl moiety, or modifications to the glycosidic bond position have been analyzed as substrates and inhibitors of *M. tuberculosis* Ado kinase. A subset of these compounds was further analyzed in human Ado kinase for the sake of comparison. Although no modifications to the ribose moiety resulted in compounds as active as Ado, the best substrates identified were carbocyclic-Ado, 8-aza-carbocyclic-Ado, and 9-[α -L-lyxofuranosyl]-adenine with 38%, 4.3%, and 3.8% of the activity of Ado respectively. The most potent inhibitor identified, 5'-amino-5'-deoxy-Ado, had a $K_i = 0.8$ μ M and a competitive mode of inhibition. MIC studies demonstrated that poor substrates could still have potent antitubercular activity.

1. Introduction

Adenosine kinase (Ado kinase) is a purine salvage enzyme that catalyzes the reaction $\text{Ado} + \text{ATP} \leftrightarrow \text{AMP} + \text{ADP}$ in a magnesium-dependent manner. Ado kinase belongs to the PfkB family of carbohydrate and nucleoside kinases, a group of proteins that catalyze the transfer of the γ phosphate of ATP to their substrates. This family includes ribokinase, hexokinases, and phosphofructokinase among its members [1]. Ado kinase activity is found in most eukaryotes, fungi, plants, and parasites, but it is seldom found in bacteria. Inhibition of Ado kinase has

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been the focus of pharmacological research in humans as well as the parasites *Toxoplasma gondii* and *Cryptosporidium parvum*. In humans, inhibition of Ado kinase results in an increase in adenosine (Ado) concentrations, and has potent anticonvulsive, analgesic, cardioprotective, and neuroprotective effects [2–4]. The pharmacologic potential for Ado kinase inhibitors against *T. gondii* and *C. parvum* parasites is due to that fact that these are both purine auxotrophs that lack a *de novo* synthesis pathway for purines [5,6]. Since Ado kinase provides the main source of purine nucleotides in these two parasites, inhibition of this enzyme leads to death by purine starvation.

Unlike *T. gondii* and *C. parvum*, *Mycobacterium spp* including *M. tuberculosis* have *de novo* synthesis pathways for nucleotides. Ado kinase activity in *Mycobacterium leprae* was reported as early as 1983 [7–9], however no further work was done to characterize the protein in *Mycobacterium spp* until 2003 [10]. Since *Mycobacterium spp* can synthesize purines *de novo*, it is unclear why they also have this salvage activity when other intracellular bacteria do not. One plausible explanation is that nucleotide synthesis is rate-limiting for the growth of *Mycobacterium spp* [11], therefore, purine and pyrimidine salvage augments the sluggish *de novo* synthesis pathway. Ado concentrations in normal human bronchoepithelial lining are much higher than circulating levels of Ado (60 μ M and <0.2 μ M, respectively) [12] and Ado is produced at high levels in the lung in response to lung hypoxia, injury, infection, and inflammation [12–16]. In asthma patients, Ado concentrations reach almost 200 μ M in bronchoepithelial fluids, largely due to mast cell stimulation and degranulation [12,13]. It is likely that the proinflammatory state incited by active tuberculosis infection results in similarly high levels of Ado, and that Ado kinase is present in *M. tuberculosis* to take full advantage of this purine reservoir. While we may never definitively answer the question as to why *M. tuberculosis* has this activity, the necessity of Ado kinase to the *Mycobacterium* genus is evidenced by the presence of this gene in *Mycobacterium leprae*, which is widely considered to have a minimal genome, retaining only the genes that are necessary for survival [17].

Characterization of *M. tuberculosis* Ado kinase revealed that this enzyme was unique among known Ado kinases [10]. Perhaps most relevantly, it differed significantly from human Ado kinase. The sequence homology that *M. tuberculosis* Ado kinase shared with other Ado kinases was low enough to prevent identification of the gene on this basis alone. Indeed, *M. tuberculosis* Ado kinase shared more sequence homology with ribokinases than other Ado kinases [10]. Most Ado kinases are monomeric proteins with a molecular mass between 35 and 40 kDa, and their activity is usually stimulated in the presence of inorganic phosphate (P_i) [15,18,19]. In contrast, Ado kinase from *M. tuberculosis* is a homodimer comprised of identical 35 kDa subunits [10,20,21]. The *M. tuberculosis* enzyme is activated in the presence of potassium but not P_i , characteristics that are more similar to ribokinases than other Ado kinases [22]. Kinetic studies with Ado, ATP, and methyl-Ado revealed that the Michaelis-Menton parameters for these reactants were significantly different between human and *M. tuberculosis* homologs [10].

Unique specificity for reactants indicated that there may be differences in the active sites for *M. tuberculosis* and human Ado kinases, which could be exploited for the development of antitubercular drugs. Nucleoside analogs occupy a unique mechanistic niche, usually requiring conversion from an inactive prodrug to an active metabolite that inhibits an essential enzyme involved in nucleic acid utilization. Interruption of nucleotide anabolism then leads to the death of the cell. Ado kinase activity may be utilized for selective phosphorylation of nucleoside analogs to metabolites active against *M. tuberculosis*. Nucleoside analogs are currently in clinical use as antiviral drugs, anticancer drugs, and gout treatments where they have a history of safety and efficacy, but they are not currently used as antibacterial drugs.

The need for antitubercular drugs with novel mechanisms of action has been heightened by the emergence of multi-drug resistant and extensively drug-resistant strains of *M. tuberculosis*, particularly in Africa and Eastern European countries [23–26]. Nucleoside analogs would complement the antitubercular drug arsenal because the mechanism of action employed by these compounds differs enough from existing antitubercular drugs that cross-resistance should be avoided. The adenosine analog, 2-methyl-Ado (methyl-Ado), provided proof of concept that nucleoside analogs have therapeutic potential against *M. tuberculosis*, with a 95% minimum inhibitory concentration (MIC) of 3 µg/ml and a 50% inhibitory concentration of 80 µg/ml in CEM cells [27–29]. Ado kinase played a central role in the mechanism of action of methyl-Ado [10,29], and knowledge about the substrate specificity for Ado kinase is required in order to successfully design other nucleoside analogs, which are more potent and selective than methyl-Ado.

In order to complement ongoing crystallographic studies of *M. tuberculosis* Ado kinase [20, 21], structure-activity studies have been performed in order to identify the requirements for the binding of Ado analogs to the Ado-binding domain [30,31]. These studies probed the adenine (Ade) moiety of the Ado-binding domain of *M. tuberculosis* Ado kinases with a total of eighty-one Ado analogs with modifications to the adenine base [30,31]. Compounds were analyzed as both substrates and inhibitors of the enzyme, and selected compounds were further analyzed in human Ado kinase. The previous structure-activity relationships (SAR) revealed the presence of hydrophobic pockets at the N¹, N⁷ and C⁸ positions of Ado, where interactions with these pockets were predictive for inhibition of the enzyme [30,31]. Furthermore, they provided a measure of the limits for the size of exocyclic modifications at the 2, 3, 7, and 8-positions that were able to bind Ado-analogs [30,31]. These results will guide the synthesis of more compounds that may be selectively phosphorylated by the *M. tuberculosis* homolog. In addition to information about the topography of the active site, the relative levels of activity and inhibition provided an approximation of the Michaelis-Menton parameters for each compound based on the Cheng-Prusoff relationship between K_i and I_{50} for the enzyme [32]. The current work complements the previous SAR by evaluating sixty-two Ado analogs with modifications to the ribose moiety alone, or in combination with substitutions to the base, in order to provide a complete picture of the Ado-binding domain of *M. tuberculosis* Ado kinase.

2. Methods and materials

2.1 Chemicals

The nucleoside analogs used in this study were obtained from several different sources. Ado, 2'-deoxy-Ado (**9**), 2'-O-methyl-Ado (**10**), and 9-[β-D-arabinofuranosyl]-adenine (**15**) were purchased from Sigma-Aldrich (St. Louis, Mo). 9-[β-L-ribofuranosyl]-adenine (**52**) was kindly provided by Dr. Mahmoud el Kouni (University of Alabama at Birmingham, Birmingham, Al.). [2,8]-³H-Ado used in the inhibition studies was purchased from Moravек Biochemicals (Brea, Ca.). All other compounds were provided by the chemical repository at Southern Research Institute (Birmingham, Alabama). Information regarding the synthesis of these compounds can be provided by contacting the corresponding author. Each compound was solubilized in water or DMSO as necessary.

2.2 Substrate assays

Substrate assays for *M. tuberculosis* Ado kinase consisted of 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM MgCl₂, 5 mM ATP, 0.01% BSA, 10 µM deoxycoformycin, and 100 µM of the appropriate test compound. Protein extracts were prepared and enzyme was purified as previously described [10]. Substrates for human Ado kinase were assayed similarly with the following changes: assay conditions consisted of 50 mM HEPES (pH 6.0), 40 mM KCl, 1 mM MgCl₂, 1 mM ATP, 0.1% BSA, 10 µM deoxycoformycin, and 100 µM of the appropriate test

compound. Human Ado kinase was prepared as previously described from clone 20-1 (a generous gift from Dr. Jozef Spychala, UNC Chapel Hill, Chapel Hill, NC) [1,31].

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. Product formation was detected 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 ten percent. Controls were run to ensure that DMSO utilized for solubility did not interfere with enzymatic activity.

2.3 Inhibition assays

Assay mixtures were identical to those for substrate assays with the addition of 0.1 µM [³H] Ado (4 µCi/ml) and 100 µM of the test compound. Inhibition assays were performed as described previously [31]. Briefly, 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, dried, and transferred to scintillation vials with 10 ml of Complete Counting Cocktail (Research Products International, Mount Prospect, Illinois). Radioactivity was detected with a Packard Tri-Carb model 1900 TR liquid scintillation analyzer, and 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 $\geq 90\%$.

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.

2.4 Determination of MIC

MIC values were evaluated in *M. tuberculosis* strains H37Ra, SRICK1, and SRICK1-pVV16/Rv2202c using a colorimetric microdilution broth assay as previously described [10,31].

2.5 Molecular modeling

All molecular modeling studies were performed on a Pentium 4 computer running the Ubuntu operating system. Pymol (DeLano Scientific LLC, San Carlos, CA) and DOCK 6.1 (Kuntz, I.D., DOCK, UCSF, San Francisco, CA) were used for construction and visualization of the models.

Protein database entries 2PKM (Adenosine Kinase complexed with 9-[α -L-lyxofuranosyl]-adenine) and 2PKN (Adenosine kinase complexed with non-hydrolysable ATP) were used as starting coordinates. For adenosine, the stereochemistry of carbon 4 was inverted, and carbon 5 was rotated prior to a single round of minimization of the ligand in the binding site pocket. The modeling of non-hydrolysable ATP analog, β,γ -methyleneadenosine-5'-triphosphate

(AMP-PCP), into the closed active site was achieved by fitting the region of the ATP binding pocket of the open structure 2PKN (16 atom pairs, RMSD 0.18Å) to that of 2PKM, and subjecting the transformed coordinates of AMP-PCP to a single round of rigid body minimization.

3. Results

Sixty-two compounds were tested that had single or multiple changes to the sugar moiety of Ado, or combinations of changes to the sugar and base (Table 1). Five compounds with modifications to the adenine base on Ado (**1a–1e**) were included for the ease of interpreting results involving multiple modifications. The structure and numbering convention for Ado is shown in Figure 1 and several of the sugar structures used in this work are shown in Figure 2.

3.1 Ribofuranosyl conformation

M. tuberculosis Ado kinase preferred the β-D-ribofuranosyl conformation or modifications that closely resembled this conformation such as carbocyclic-Ado (**3**, Figure 2e). The L-enantiomer, 9-[β-L-ribofuranosyl]-adenine (Fig 2b, 42), was neither a substrate, nor an inhibitor, indicating that it was not recognized by the active site. Furthermore, the α-isomer, 9-[α-D-ribofuranosyl]-adenine (Fig 2c, 43), was not a substrate, but was a weak inhibitor, indicating that it at least bound poorly to the active site. Ado kinase only phosphorylated sugars in the furan conformation and excluded all twelve pyranoses tested (**52–63**). Furthermore, the acyclo-adenosine analogs, 9-(2-hydroxyethoxymethyl)-adenine (acyclo-adenosine, **50**) and 9-[2-[(2-hydroxyethyl)-methylamino]-ethoxymethyl]-adenine (**51**), were not substrates or inhibitors of *M. tuberculosis* Ado kinase. Following is a summary of the results of changes to the ribofuranosyl moiety.

3.2 2'-position

Consistent with previous observations with human Ado kinase [33–35], 2'-deoxy-Ado (**9**) was a poor substrate for *M. tuberculosis* Ado kinase. The enzyme had a preference for the 2'-hydroxyl moiety to be present and *trans* to the adenine moiety as illustrated by the <99% decrease in activity seen with both 2'-deoxy-adenosine (**9**) and 9-[β-D-arabinofuranosyl]-adenine (araA, **15**, Fig 2f). Several compounds with 2'-modifications were substrates, albeit at low levels. The most active substrates were 2'-deoxy-2,2'-difluoro-Ado (**13**), araA (**15**) and 2-fluoro-araA (**16**). When substitutions to the base are combined with substitutions at the 2'-position as in the case of 2-fluoro-araA (**16**) and 2'-deoxy-2,2'-difluoro-Ado (**13**), the resulting activity is closer to the activity seen with a single change at the 2'-position than an intermediate value. Also, addition of a methyl group to the 2'-hydroxyl group (**10**) resulted in activity of <1 nmol/mg-min, indicating that substitutions to the 2'-hydroxyl are poorly tolerated.

2-Chloro-2'-deoxy-2'-fluoro-Ado (**12**) was the only compound with a 2'-substitution that demonstrated noteworthy inhibition with 13% of inhibition at 10 μM. This inhibition was similar to that previously determined for 2-chloro-adenosine [31]. All other compounds with modifications at the 2'-position were either poor inhibitors or did not inhibit *M. tuberculosis* Ado kinase at all.

3.3 3'-position

For *M. tuberculosis* Ado kinase, the presence of a 3'-hydroxyl group *trans* to the adenine moiety was a requirement for substrate activity. Modification of the 3'-hydroxyl group had a greater impact on substrate activity than modification of the 2'-hydroxyl group for this enzyme. Replacing the 3'-hydroxyl group with an amino or azido group (**21** and **22**) abolished activity and 3'-deoxy-Ado (**20**) had no measurable activity; each of these had substrate activity of <1 nmol/mg-min. Whereas, changing the 3'-hydroxyl group to the *cis*-conformation, as in the case

of 9- $[\beta$ -D-xylofuranosyl]-Ade (Fig 2g, 23), resulted in a specific activity of 1 nmol/mg-min. Furthermore, 9- $[\beta$ -D-3-ethynyl-ribofuranosyl]-2-methyladenine, which has a 3'-ethynyl group (**26**, 3'-C \equiv CH \uparrow) was not a substrate for *M. tuberculosis* Ado kinase.

These results stood in contrast to human and mammalian Ado kinases that were more permissive to modifications to the 3'-position, such as 3'-deoxyadenosine or 3'-aminoadenosine, than of modifications to the 2'-position, such as araA and 2'-deoxyadenosine [33,35–37].

No compounds with combinations of 2' and 3' substitutions (**27–29**) were either substrates or inhibitors of *M. tuberculosis* Ado kinase and none of the multiple changes involving the base and the 3'-position resulted in active compounds (**24–26**). No compounds with substitutions or modifications at the 3'-position were inhibitors. The presence of a 3'-hydroxyl group *trans* to the adenine moiety is important both for substrate recognition and the binding of inhibitors, suggesting that steric hindrance may play a role at this site.

3.4 4'-oxygen

The 4'-oxygen position was the most flexible among all of the substitutions made to the ribose moiety for both human and *M. tuberculosis* Ado kinases. Carbocyclic-Ado (aristeromycin, Fig 2e, 3) maintained about the same activity in both human and *M. tuberculosis* homologs (37% and 38% of the activity of Ado respectively). Although 2-amino-Ado was similarly active in both Ado kinases [31], 2-amino-carbocyclic-Ado (**4**) was at least a 20-fold better substrate for *M. tuberculosis* Ado kinase than human. The double-substituted 8-aza-carbocyclic-adenosine (**6**) was less active than carbocyclic-Ado (**3**), but was the second-best substrate in this series with a specific activity of 170 nmol/mg-min. Although 8-aza-carbocyclic-adenosine (**6**) exhibited good substrate activity in *M. tuberculosis*, this compound was more active in human Ado kinase. Activities measured with human Ado kinase were consistent with previous reports [35,36,38,39].

Substitution of the 4'-oxygen with a 4'-sulfur atom, as is the case with 2-chloro-4'-thio-adenosine (**2**, <1nmol/mg-min), resulted in at least a 460-fold decrease in substrate activity over its parent, 2-chloro-adenosine (**1b**, 460 nmol/mg-min) [31]. Since sulfur atoms have similar electronegativity and have valence electrons more similar to oxygen than carbon atoms, and carbocyclic-adenosine (**3**) is a better substrate than the 4'-thio-adenosine analog (**2**), it is unlikely that the loss of activity is due to an inability to bind this site. This loss of activity is more likely due to the larger size of the sulfur atom, which may negatively effect the puckering of the ribose moiety. Therefore, the size of the substitution at the 4'-position may be a determinate of substrate activity for this site.

All of the carbocyclic compounds (**3–8**) were poor inhibitors, inhibiting Ado phosphorylation by no more than 81% at 100 μ M.

3.5 5'-position

The 5'-position was also permissive to substitution, possibly because it is the site of catalysis and therefore needs room for the phosphorylation reaction to take place. The *cis*-conformation of the 5'-C was not an absolute requirement for either human or *M. tuberculosis* Ado kinase as demonstrated by 9- $[\alpha$ -L-lyxofuranosyl]-adenine (Fig 2d, 34) with 4% and 15% of the activity of Ado in *M. tuberculosis* and human enzymes respectively. This compound differs from Ado only in that the 5'-C is *trans* to the adenine moiety. The addition of a 2-fluorine atom to this compound (9- $[\alpha$ -L-lyxofuranosyl]-2-fluoro-adenine, **35**) resulted in a compound with similar activity in *M. tuberculosis* Ado kinase, but greatly diminished activity in the human homolog (0.8 nmol/mg-min). One report exists where 9- $[\alpha$ -L-lyxofuranosyl]-adenine (**34**) was

determined to be an inhibitor, but not a substrate for human Ado kinase [4]. However, the substrate activity that we measured for 9-[α -L-lyxofuranosyl]-adenine (**34**) in human Ado kinase was similar to previous reports in human and mammalian Ado kinase [35,36]. Other compounds with modifications to the 5'-position, including 5'-amino-5'-deoxy-adenosine (**32**), 9-[β -D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine (**36**), and 9-[β -D-5-methyl-(*talo*)ribofuranosyl]-2-fluoro-adenine (**37**) resulted in very low substrate activity for human Ado kinase, maintaining at most 0.3% of the activity of Ado. Although they were both poor substrates for *M. tuberculosis* Ado kinase, 9-[β -D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine (**36**) was about twice as active as 9-[β -D-5-methyl-(*talo*)-ribofuranosyl]-2-fluoro-adenine (**37**). The difference between these two stereoisomers is that the 5'-methyl group in the *allo*-conformation points towards the back of the plane of the ribose moiety in the direction of the 4'-oxygen, while in the *talo*-conformation it points forward toward the 2'-hydroxyl group. Neither of these compounds had measurable substrate activity in human Ado kinase.

While it is known not to be a substrate for mammalian Ado kinases [40–42], there was strong evidence that 5'-amino-5'-deoxy-Ado (**32**) was a substrate for *M. tuberculosis* Ado kinase. With the *M. tuberculosis* enzyme, a peak appeared in the region consistent with the formation of a monophosphate, but the peak area did not increase linearly with time. The ADP peak increased with time. Since ADP is also a product of the reaction, the increase of this product provided indirect evidence that 5'-amino-5'-deoxy-Ado (**32**) was a substrate for *M. tuberculosis* Ado kinase. The appearance of the monophosphate peak and the increase of the ADP peak were magnesium-dependent and did not appear in the presence of iodotubercidin, a potent inhibitor of Ado kinase, indicating that product formation was due to Ado kinase activity and not due to a possible contaminating enzyme. Although a peak appeared in the region where one would expect a nucleoside monophosphate, we have not yet verified that the peak was 5'-amino-5'-deoxy-AMP (Fig 3). The nucleoside apparently fluctuated between the parent compound and the monophosphate. It was not further metabolized because the sum of these two peaks was constant with time. The fluctuation of the monophosphate peak led us to believe that the product of the reaction was unstable. Since the ADP peak increased with time, we ruled out a reversible reaction which would have consumed this product as well. It was uncertain whether the disappearance of the product was due to innate instability or assay conditions, since we terminated the reaction by the addition of 1M perchloric acid. Terminating the reaction by the addition of EDTA or iodotubercidin did not change the results, and the product never disappeared altogether. Therefore, we believe that the product is unstable under our assay conditions.

The activity of 5'-amino-5'-deoxy-Ado (**32**) is noteworthy because this compound is known not to be a substrate for human Ado kinase; indeed, it is a potent inhibitor of Ado kinases [40–43]. The fact that the 5'-hydroxyl group is the site of catalysis for this enzyme makes this compound more intriguing than changes to other sites. If the peak that we have observed is the monophosphate product, it indicates that there are major differences at the catalytic site between human and *M. tuberculosis* Ado kinases. Further studies of 5'-amino-5'-deoxy-adenosine (**32**) and its phosphorylated product are warranted in light of these unusual results.

The 5'-position provided the most potent inhibitors of all of the sugar substitutions. Of these, 5'-amino-5'-deoxy-Ado (**32**) was the best competitive inhibitor with a K_i of $0.8 \pm 0.4 \mu\text{M}$. This compound was more potent at inhibiting Ado phosphorylation than 5'-deoxy-Ado (**30**). Furthermore, compounds that had the addition of a 5'-methyl group in the *talo*-conformation (**37 and 39**) were about 10-times less potent as inhibitors than their *allo*-conformers (**36 and 38**).

3.6 Glycosidic bond position

Four compounds were evaluated which had multiple changes, at least one of which involved the position of the glycosidic bond. Three of these, 7-[α -D-ribofuranosyl]-adenine (**45**, Fig 2c), 7-[α -D-arabinofuranosyl]-adenine (**46** similar to Fig 2c, but with the 2'-OH \uparrow), and 8-aza-8-[β -D-ribofuranosyl]-adenine (**48**) had no measurable substrate activity and all were poor inhibitors of Ado kinase. The fourth compound, 3-[β -D-ribofuranosyl]-adenine (**47**), demonstrated both substrate and inhibitor activity with a specific activity of 28 nmol/mg-min (0.7% of the activity of Ado). This compound, however, was also a much better substrate for human Ado kinase, with a specific activity of 268 nmol/mg-min (11% of the activity of Ado).

Previous studies of Ado analogs with substitutions at the 3-position of Ado indicated that steric hindrance may inhibit the binding of substrates to the active site [30]. The result that we found with 3-[β -D-ribofuranosyl]-adenine (**47**) seems to contradict this theory. One possible explanation for this difference may be that the series of compounds evaluated in the previous study were 3-deaza-adenosine analogs. In the current study, the nitrogen atom at the 3-position remains intact, indicating that this nitrogen likely plays an important role in substrate recognition. That 3-[β -D-ribofuranosyl]-adenine (**47**) fits well into the active site is supported not only by its substrate activity, but also by its ability to inhibit Ado kinase at the 10 μ M level.

3.7 MIC assays

MIC values were determined in *M. tuberculosis* H37Ra, in an Ado kinase-deficient strain derived from H37Ra (SRICK1), and in SRICK1 complemented with the *adoK* gene (SRICK1::*adoK*) using three nucleoside analogs (Table 2). Each of the compounds selected had modifications at the 5'-position with substrate activity ranging from 4 to 130 nmol/mg-min. Of the compounds tested, 9-[β -D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine (**36**) was the most promising in terms of antimycobacterial activity, with activity similar to the ethambutol control. The specific activity of the compound as measured by our assays was not predictive for efficacy against bacteria. Indeed, 9-[α -L-lyxofuranosyl]-2-fluoro-adenine (**35**) was not as effective against the intact organism as 9-[β -D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine (**36**), even though 9-[α -L-lyxofuranosyl]-2-fluoro-adenine (**35**) was a much better substrate for Ado kinase. Although they were active at different levels, both compounds exerted their antimycobacterial activity in an Ado kinase-dependent manner as evidenced by the lack of activity in SRICK1, and restoration of activity in SRICK1::*adoK*. The lack of antitubercular activity seen with 5'-amino-5'-deoxy-adenosine (**32**) is consistent with its unusual substrate activity; this compound was included specifically because of its unusual activity and modifications at the site of catalysis.

4. Discussion

M. tuberculosis Ado kinase is of interest because it is the first prokaryotic Ado kinase to be characterized and preliminary characterization demonstrated that this enzyme is unique among Ado kinases [10]. In addition to its role in purine salvage, Ado kinase can also be used as a selective filter for phosphorylation of subversive substrates with antimycobacterial activity. This SAR was performed as part of an effort to rationally design compounds that will be subversive substrates for Ado kinase from *M. tuberculosis*. When modeled against a high-resolution crystal structure, these studies will identify the required elements of a pharmacophore for Ado kinase and to lead future compound design. A previous SAR explored the Ade moiety of Ado and provided insight into the requirements for the binding of substrates and inhibitors to the active site [31]. The current work complemented the previous study by similarly studying structural modifications to the ribose moiety of Ado that would result in phosphorylated products or inhibition of enzymatic activity.

Very few modifications to the ribofuranosyl moiety resulted in active substrates or inhibitors; the furan conformation had to be maintained as none of the sugars in the pyran conformation (**52–63**) or acyclo-adenosine analogs (**50 and 51**) were substrates. In contrast to the SAR involving modifications to the adenine base in which 32 of the 80 compounds tested (40%) had measurable substrate activity and 69 of the 80 were inhibitors (86%), in the current study involving modifications to the ribofuranosyl moiety only 14 out of 63 (22%) of the compounds tested were substrates and 28 of 63 (44%) were inhibitors. The strict conservation of the ribofuranosyl moiety may be due to the structural similarity that this enzyme shares with ribokinase, and further bolsters the argument that this activity may have arisen in *Mycobacterium spp* as a result of convergent evolution [10,21,22]. Ribokinase is a member of the PfkB family of carbohydrate kinases along with Ado kinase, inosine-guanosine kinase, fructokinase, 1-phosphofructokinase, 6-phosphofructokinase, and other enzymes [44,45]. Like Ado kinase from *M. tuberculosis*, ribokinase exists as a homodimer, and optimum kinase activity occurs in the presence of Mg^{+2} and K^{+} , with an optimum pH range similar to that of *M. tuberculosis* Ado kinase, pH 8–9 [10]. The best substrates for ribokinase are D-ribose and 2-deoxy-D-ribose, with k_{cat} values of 86 and 27 s^{-1} , respectively [46]. Other substrates for ribokinase include D-arabinose, D-xylose, and D-fructose, with respective k_{cat} values of 0.64, 0.92, and 0.24 s^{-1} . The overwhelming preference of ribokinase for D-ribose mirrors the selectivity of *M. tuberculosis* Ado kinase for β -D-ribofuranoses.

The high-resolution crystal structure of *M. tuberculosis* Ado kinase that was recently published helped to explain some of the observations made in the current work (Figure 4) [20]. For example, the crystal structure revealed a pocket with a diameter of $\sim 6.5\text{\AA}$ adjacent to the 2-C of Ado that permits substitutions at the 2-position like 2-fluoro-Ado (**1a**) and 2-methyl-Ado (**1c**) (Figure 4B). Furthermore, the conservation of the ribofuranosyl moiety has its origins in the protein structure. Asp12, and Asn52 hydrogen bond to the O3', while Phe102 may provide steric hindrance that prohibits the addition of a moiety at the 3-position on the cis side of the base like 9- $[\beta$ -D-xylofuranosyl]-adenine (**23**) (Figure 4A). Hydrogen bonding may occur at the 2'-OH due to interactions with Gly48 and Asp12 when the 2'-OH is trans to the base, however this SAR demonstrated the ability to make small additions at the 2'-C cis to the adenine base like 9- $[\beta$ -D-arabinofuranosyl]-adenine (araA, **15**), 2-fluoro-araA (**16**), and 2'-deoxy-2,2'-difluoro-adenosine (**12**). In these and other cases, the crystal structure supports the results that we observed with our SAR studies.

The actual mechanism by which the γ -phosphate of ATP is transferred to Ado has yet to be elucidated. In other Ado kinases, including human, this transfer involves the deprotonation of the 5'-hydroxyl group by an aspartic acid residue, followed by a water-mediated nucleophilic attack of the γ -phosphate by the negatively charged O-5' atom, and the products, AMP and ADP are formed via an SN_2 reaction [20,47]. Although the same mechanism has not yet been empirically proven for Ado kinase from *M. tuberculosis*, all of the players are in place, including the aspartic acid residue (Asp 257, in *M. tuberculosis* Ado kinase), two water molecules, and an anion hole near the γ -phosphate of ATP (Figure 4C) [20]. One major difference in the mechanism of phosphorylation between the human and *M. tuberculosis* enzymes involves the binding of ATP to the active site. In the human enzyme, the binding of Ado to the active site induces a conformational change that optimizes the configuration of the anion hole to accommodate ATP. There is no evidence that *M. tuberculosis* Ado kinase undergoes a similar conformational change upon binding ATP [20].

Integrating the results of this SAR with previous studies of modifications to the Ade moiety, a recurring result was that a combination of a substitution such as a fluorine atom or amino group at the 2-position with a modification at another site on either the base or sugar moieties resulted in compounds that were more selective for *M. tuberculosis* than human Ado kinase (Table 3). This held true even when the substitution was a fluorine atom, although as a single

substitution 2-fluoro-Ado was more active in human than in *M. tuberculosis* Ado kinase [31]. The compound sets carbocyclic-Ado (**3**) vs 2-amino-carbocyclic-Ado (**4**), araA (**15**) vs 2-fluoro-araA (**16**), and 9-[α -L-lyxofuranosyl]-adenine (**34**) vs 9-[α -L-lyxofuranosyl]-2-fluoro-adenine (**35**) illustrate this point best. For compounds that exhibit substrate activity in both enzymes, addition of a small, exocyclic substitution at the 2-position improves selectivity for the *M. tuberculosis* enzyme and reduces the possibility that the compound will be a substrate for Ado deaminase. Two of the major challenges of developing Ado analogs as antitubercular drugs are attempting to circumvent catabolism by host enzymes like Ado deaminase, and to avoid host toxicity. One example of this is the liberation of 2-fluoro-adenine by methylthioadenosine phosphorylase (MTAP). Some of our most promising compounds, including 9-[α -L-lyxofuranosyl]-2-fluoro-adenine (**35**) and 9- [β -D-5-methyl-(*talo*)-ribofuranosyl]-2-fluoro-adenine (**37**), are known substrates for this host-associated protein [48]. In addition to MTAP, there are a multitude of host and bacterial enzymes that utilize nucleosides and nucleotides that could result in inactivation of the compound or host toxicity.

Although the ribofuranosyl moiety offered fewer opportunities for modification than the adenine moiety, the results are still promising for drug development (results are summarized in Table 4). Even compounds that are good substrates may not have antitubercular activity if they do not inhibit a critical downstream target, or if they are metabolized by other enzymes in the purine pathway. Conversely, MIC studies illustrated that compounds need not be excellent substrates in order to exert an antimycobacterial effect in an Ado kinase-dependent manner. Indeed, 9-[α -L-lyxofuranosyl]-2-fluoro-Ade (**35**) and 9-[β -D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-Ade (**38**) both had Ado kinase-dependent antimycobacterial activity even though their substrate activity was low. As long as the phosphorylated product is potent enough to inhibit a critical downstream target, a poor Ado kinase substrate may still have antitubercular activity. For active substrates, MIC studies like the one presented here will give insight into whether or not Ado kinase is involved in the mechanism of action of the compound. This SAR was critical for completing studies of the Ado binding domain of *M. tuberculosis* Ado kinase, and permitting the design and synthesis of more active substrates.

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Abbreviations

Ade	adenine
Ado	adenosine
AD	adenosine deaminase
araA	9-[β -D-arabinofuranosyl]-adenine
AMP-PCP	

	β,γ -methyleneadenosine-5'-triphosphate
BSA	bovine serum albumin
DTT	dithiothreitol
formycin A	8-aza-9-deaza-Ado
IPTG	isopropyl- β -D-thiogalactopyranoside
methyl-Ado	2-methyl-adenosine
MTAP	methylthioadenosine phosphorylase
MIC	95% minimum inhibitory concentration
PBS	phosphate buffered saline
P_i	inorganic phosphate
PMSF	phenylmethylsulfonyl fluoride
SAR	structure-activity relationship

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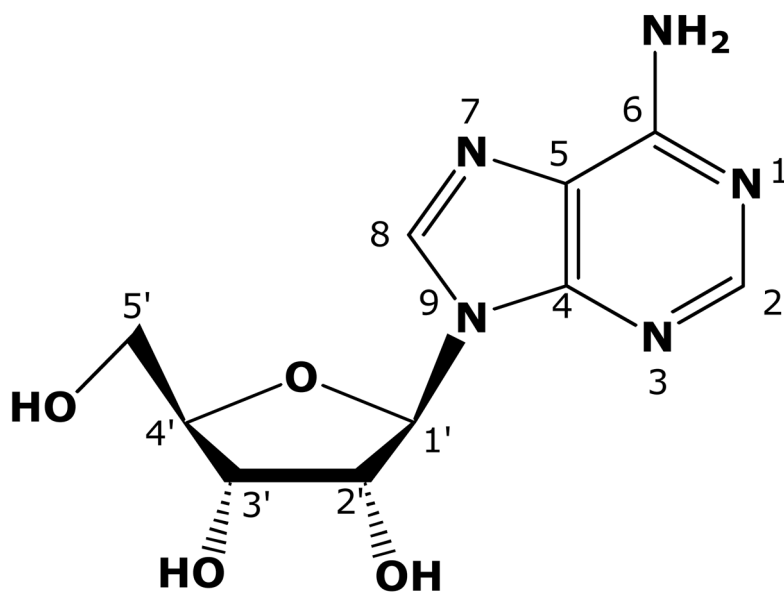


Figure 1.
Structure and numbering convention for Ado.

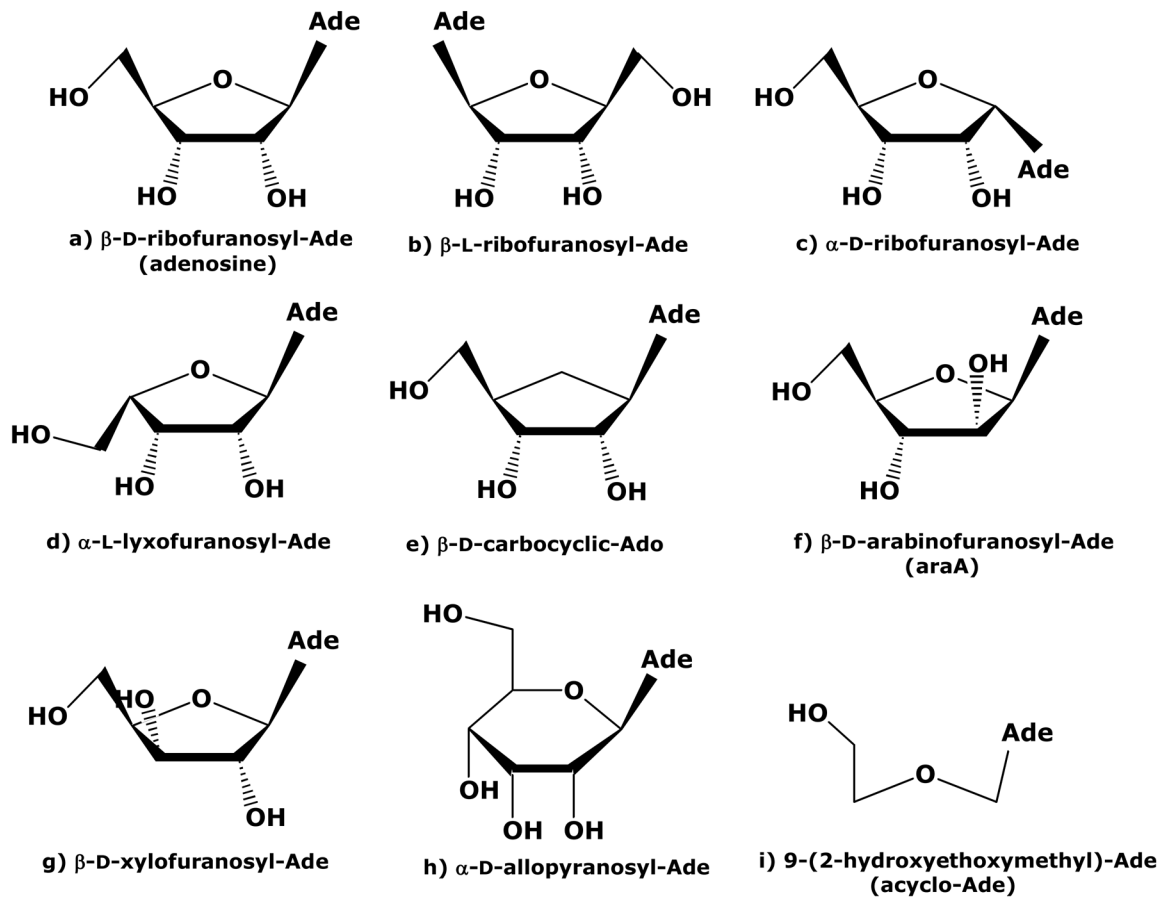


Figure 2.
Structures of sugar moieties utilized in this structure-activity relationship.

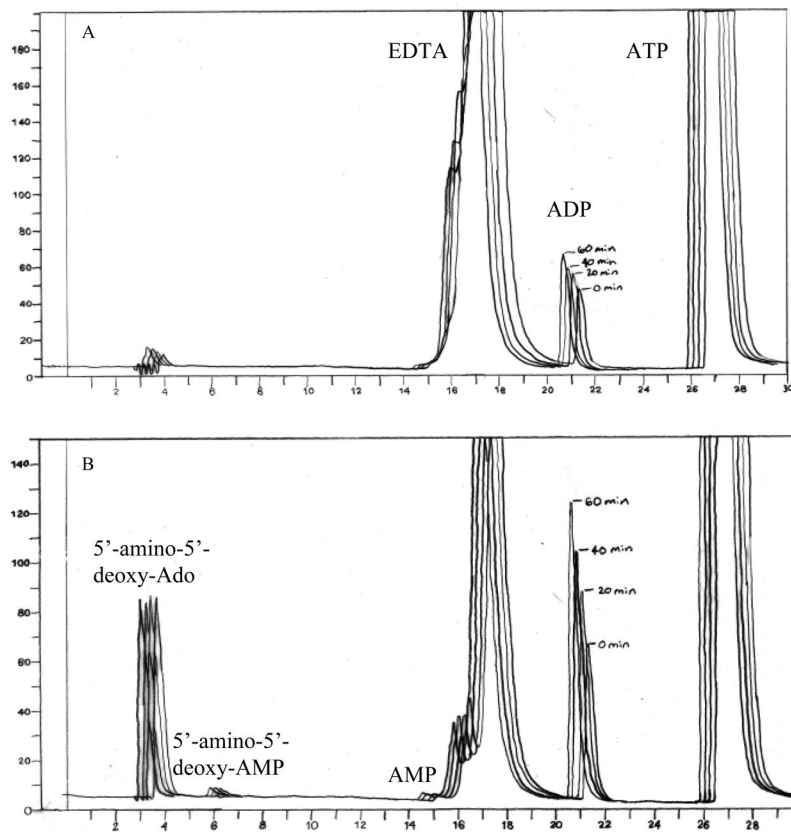
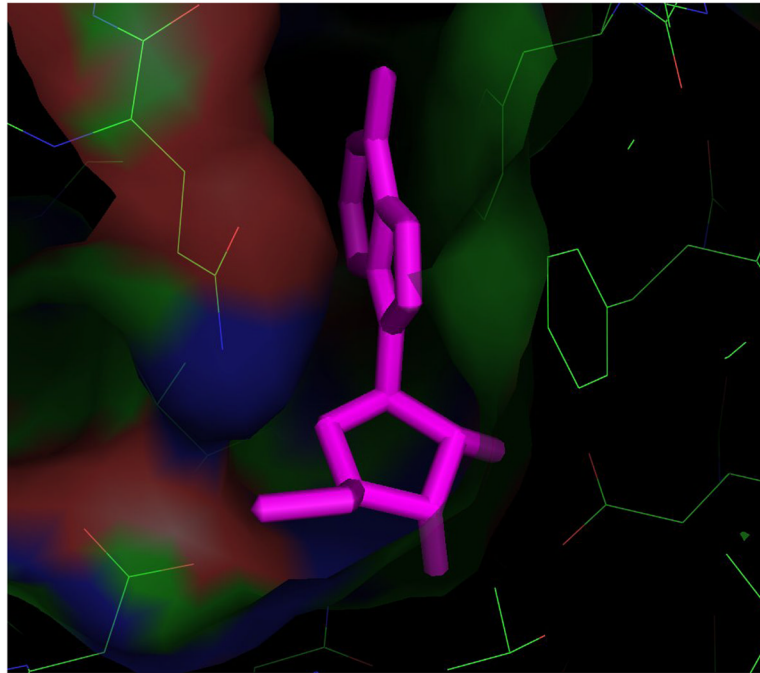


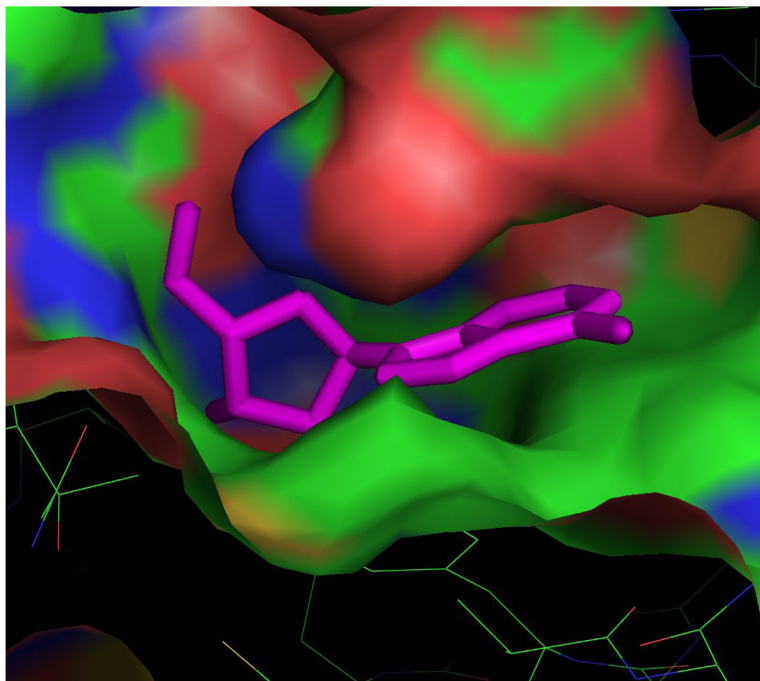
Figure 3. BioBasic anion exchange chromatography of an Ado kinase assay with 5'-amino-5'-deoxyadenosine as a substrate

Activity assays were performed as described in the materials and methods section. Two μg of protein were used per 50 μl sample. Reactions were stopped by the addition of 10 mM EDTA prior to perchloric acid extraction. Aliquots of 50 μl were taken at 0, 20, 40, and 60-min. A) A negative control assay contains everything except 5'-amino-5'-deoxyadenosine. B) A typical assay with 5'-amino-5'-deoxyadenosine.

A)



B)



C)

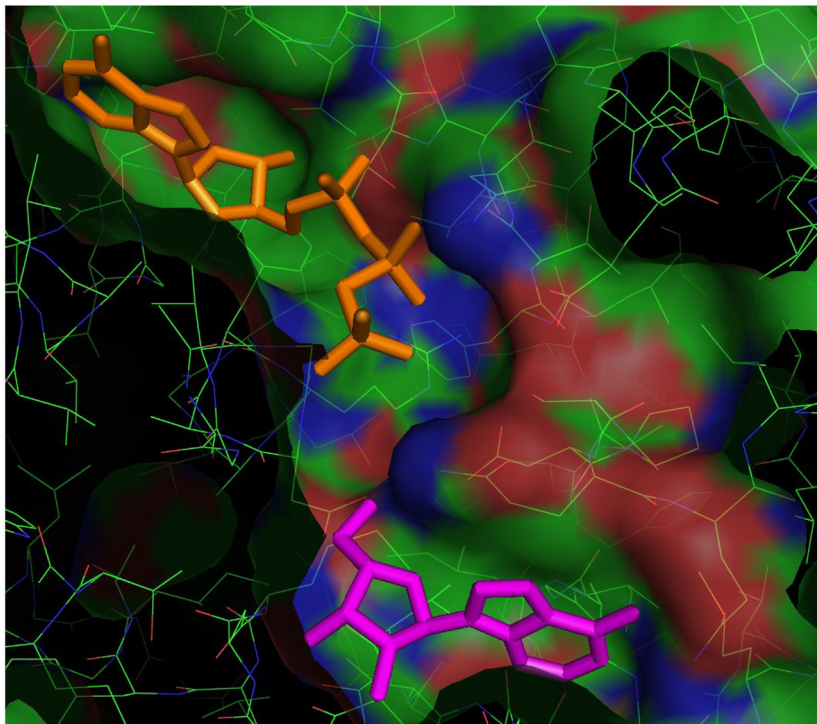


Figure 4. Ligands docked in the active site of Ado kinase

A) Adenosine (magenta) modeled into the active site from the perspective of the ribofuranosyl moiety. Phe 102 (right of center) forms the upper surface of the pocket where the ribofuranosyl moiety nests, while Asp 12 forms hydrogen bonds with the 2' and 3' hydroxyl groups (lower right). B) One of the better substrates, 9-[α -L-lyxofuranosyl]-adenine (**34**, Fig 2d) from a top-down perspective of looking downward from C1 and N7 of the adenine base. The area above this region is open and available for modifications. The yellow lining the pocket near the 2-position of the Ade base is Met 121, this is the pocket in which a 2-methyl or 2-fluoro group would rest. The 2' and 3'-hydroxyl groups are clearly nested within the ribofuranosyl pocket, and there is plenty of room to rotate the 5'-hydroxyl so that it is trans to the adenine base, as in the case of 9-[α -L-lyxofuranosyl]-adenine (shown). C) This image depicts 9-[α -L-lyxofuranosyl]-adenine (magenta) modeled with AMP-PCP (orange), a non-hydrolysable form of ATP, in the active site of Ado kinase, as they would appear when Ado kinase is in a closed conformation. The γ -phosphate of AMP-PCP is nestled within the anion hole in the active site.

Table 1
Effect of modifications of the ribofuranosyl moiety of Ado on Ado kinase activity

	Compound name	<i>M. tuberculosis</i> Specific activity (nmol/mg-min)	Inhibition ^a	K_i^b (μ M)	Human Specific activity (nmol/mg-min)	Inhibition ^a
1	Adenosine (9- β -D-ribofuranosyl]-adenine)	4000 \pm 450			2400 \pm 750	
1a	2-fluoro-adenosine ^c	2070 \pm 430	+++	0.5 c	1800 \pm 220	+
1b	2-chloro-adenosine ^c	460 \pm 60	++		16 \pm 3	
1c	2-methyl-adenosine ^c	74 \pm 2	++		4 \pm 0.6	
1d	6-methyl-purine riboside ^c	110 \pm 6	++		980 \pm 100	
1e	8-aza-adenosine ^c	160 \pm 50	++		910 \pm 400	
	4'-oxygen position					
2	2-chloro-4'-thio-adenosine	<1	+			
3	Carbocyclic adenosine (aristeromycin)	1500 \pm 230	+		860 \pm 220	
4	2-amino-carbocyclic-adenosine	17 \pm 3	-		<2	
5	7-deaza-carbocyclic-adenosine	<1	+			
6	8-aza-carbocyclic-Ado	170 \pm 20	+		620 \pm 194	+
7	2-amino-8-aza-2'-deoxy-carbocyclic-adenosine	<6	-			
8	2-amino-6-hydroxymethyl-8-aza-carbocyclic-adenosine	<2	-			
	2'-position					
9	2'-deoxy-adenosine	13 \pm 2	+		73 \pm 11	
10	2'-O-methyl-adenosine	<1	+			
11	2-chloro-2'-deoxy-adenosine	<1	+			
12	2-chloro-2'-deoxy-2-fluoro-adenosine	<1	++			
13	2'-deoxy-2,2'-difluoro-adenosine	29 \pm 3	++		<1	
14	8-aza-2'-deoxy-adenosine	<2	+			
15	9- β -D-arabinofuranosyl]-adenine (araA)	23 \pm 2.3	-		6 \pm 0	
16	2-fluoro-araA	36 \pm 3	+		<1	
17	2-chloro-2'-deoxy-2-fluoro-araA	<1	+			
18	1,4'-anhydro-2'-deoxy-D-araA	<1	-			
19	1,4'-anhydro-2'-deoxy-6-chloro-D-araA	<3	-			
	3'-position					
20	3'-deoxy-adenosine (cordycepin)	<1	-			-
21	3'-deoxy-3'-amino-adenosine	<1	-			+
22	3'-deoxy-3'-azido-adenosine	<1	-			
23	9- β -D-xylofuranosyl]-adenine	1 \pm 0.1	-			
24	9- β -D-xylofuranosyl]-6-methylpurine	<1	-			
25	9- β -D-xylofuranosyl]-6-oxy-8-azapurine	<7	-			
26	9- β -D-3-ethyl-ribofuranosyl]-2-methyladenine	<1	-			
	2'- and 3'-positions					
27	9- β -D-2-azido-2'-deoxy-xylofuranosyl]-adenine	<1	-			
28	2-chloro-2',3'-O-isopropylidene-adenosine	<1	-			
29	8-aza-2',3'-O-isopropylidene-adenosine	<3	-			
	5'-position					
30	5'-deoxy-adenosine	<1	++			+++
31	5'-carboxamido-adenosine	<1	+			
32	5'-amino-5'-deoxy-adenosine	?	+++	0.8 c	<1	+++
33	9- β -D-glucofuranosyl]-2-methyladenine	<1				
34	9- α -L-lyxofuranosyl]-adenine	150 \pm 24	+		360 \pm 150	
35	9- α -L-lyxofuranosyl]-2-fluoro-adenine	130 \pm 16	+		0.8 \pm 0.2	
36	9- β -D-5-methyl-(<i>allo</i>)-ribofuranosyl]-2-fluoro-adenine	4 \pm 0.3	++		<0.5	
37	9- β -D-5-methyl-(<i>allo</i>)-ribofuranosyl]-2-fluoro-adenine	1.5 \pm 0.06	++			
38	9- β -D-5-methyl-(<i>allo</i>)-ribofuranosyl]-6-methyl-purine	<2	++			
39	9- β -D-5-methyl-(<i>allo</i>)-ribofuranosyl]-6-methyl-purine	<1	+			

Compound name	<i>M. tuberculosis</i> Specific activity (nmol/mg-min)	Inhibition ^a	K_i^b (μ M)	Human Specific activity (nmol/mg-min)	Inhibition ^a
40 8,5'-(R)-cycloadenosine	<1	-			
41 8-aza-5'-deoxy-5'-methylsulfonyl-adenosine	<2	-			
Multiple positions					
42 9-[β -L-ribofuranosyl]-adenine	<1	-			
43 9-[α -D-ribofuranosyl]-adenine	<1	+			
44 9-[α -D-lyxofuranosyl]-adenine	<1	-			
45 7-[α -D-ribofuranosyl]-adenine	<2	+			
46 7-[α -D-arabinofuranosyl]-adenine	<2	+			
47 3-[β -D-ribofuranosyl]-adenine	28 \pm 2	++	268 \pm 41		
48 8-aza-8-[β -D-ribofuranosyl]-adenine	<1	+			
49 2',3',5'-tri-O-acetyl-adenosine	<1	+			
50 9-(2-hydroxyethoxymethyl)-adenine (Acyclo-adenosine)	<1	-		-	
51 9-[2-[(2-hydroxyethyl)-methylamino]-ethoxymethyl]-adenine	<1	-		-	
Pyranoses					
52 9-[β -D-ribofuranosyl]-adenine	<1	-			
53 9-[β -D-allopyranosyl]-adenine	<1	-			
54 9-[β -D-fructopyranosyl]-adenine	<1	-			
55 1-(adenyl-9-yl)-1-doxy- β -D-glucopyranamide	<1	-			
56 9-(2-deoxy- β -D-erythro-pentopyranosyl)-adenine	<1	+			
57 9-(2-deoxy- α -D-erythro-pentopyranosyl)-adenine	<1	-			
58 9-(3-azido-3,4-dideoxy- β -D-erythro-pentopyranosyl)-adenine	<1	-			
59 9-[β -L-galactopyranosyl]-adenine	<1	-			
60 9-[6-O- α -D-galactopyranosyl]- β -D-glucopyranosyl]-adenine	<1	-			
61 9-[α -D-mannopyranosyl]-adenine	<1	-			
62 9-[α -D-talopyranosyl]-adenine	<1	-			
63 6-(adenyl-9-yl)-tetrahydropyran-2-methanol	<1	-			

^aInhibition of 0.1 μ M adenosine phosphorylation is indicated as follows: '-', <10% inhibition at 100 μ M of compound; '+', 10–90% inhibition at 100 μ M; '++', 10–90% inhibition at 10 μ M; '+++', 10–90% inhibition at 1 μ M.

^bThe manner of inhibition is denoted by a 'c' for competitive inhibition.

^cThese previous results were included for ease of comparison with the current study [32].

Table 2

MIC results with selected nucleosides

Compound Name	specific activity (nmol/mg-min)	H37Ra	SRICKI	SRICKI::adoK
5'-amino-5'-deoxy-Ado	ND ^b	>100	>100	>100
9- β -D-5-methyl-(<i>allo</i>)-ribofuranosyl]-2-fluoro-adenine	4	1-10	>50	0.1-1
9-[α -L-lyxofuranosyl]-2-fluoro-adenine	1.30	10-100	>100	10-100

^a MIC assays were performed at least twice for each compound.^b ND specific activity was not determined for this compound.

Table 3
Effect of exocyclic 2-substituted Ado analogs on enzyme selectivity

Compound	Ado kinase Specific Activity (% of Ado control)		Ratio ^a
	<i>M. tuberculosis</i>	Human	
<i>Single substitutions:</i>			
Adenosine	100	100	1
2-Fluoro-Ado ^b	52	75	0.7
2-Chloro-Ado ^b	12	0.7	17
2-Amino-Ado ^b	6	5	1.2
2-Methyl-Ado ^b	1.9	0.2	9.5
<i>Effect of multiple substitutions:</i>			
3-deaza-Ado ^c	0.03	<0.04	>0.8
2-Fluoro-3-deaza-Ado ^c	1.7	<0.04	42
8-aza-Ado ^b	4	38	0.1
2-Fluoro-8-aza-Ado ^b	3.8	9	0.4
Carbocyclic-Ado	38	36	1.1
2-Amino-carbocyclic-Ado	0.4	<0.08	>5
araA	0.6	0.2	3
2-Fluoro-araA	0.9	<0.04	>22
9-[α -L-Lyxofuranosyl]-adenine	3.8	15	0.2
2-Fluoro-9-[α -L-Lyxofuranosyl]-adenine	3.2	0.03	107
Formycin A ^b	95	25	3.8
2-Fluoro-formycin A ^b	55	<0.04	>1400
2-Amino-formycin A ^b	45	0.6	75

^aThe ratio is calculated as (% of Ado control) for *M. tuberculosis*/human. A ratio of >1 is more selective for *M. tuberculosis* Ado kinase while a ratio of <1 is more selective for human Ado kinase.

^bValues are adapted from a previous study of modifications to the adenine base of Ado [32].

^cValues are adapted from a previous study of 3-deaza-Ado analogs [31].

Table 4
Summary of results of modifications to the ribofuranosyl moiety

Furan conformation There was an absolute requirement for the sugar moiety to be in a furanosyl-like conformation. Neither of the acyclo-adenosine analogs tested (50–51), nor any of the twelve pyranoses assayed (52–63) were substrates for Ado kinase. It is unlikely that many of these compounds were recognized by the active site despite the presence of the adenine base as evidenced by the fact that only 9- (2-deoxy- β -D-*erythro*-pentopyranosyl)-adenine (56) poorly inhibited the phosphorylation of 0.1 μ M Ado at 100 μ M. None of the other pyranoses or acyclo-Ado analogs were either substrates for, or inhibitors of, *M. tuberculosis* Ado kinase. Only modifications that maintained a β -D-ribofuranosyl-like conformation were substrates for *M. tuberculosis* Ado kinase.

2' A trans-2'-hydroxyl group was preferred, although a cis-2'-hydroxyl group (araA, 15) and 2'-deoxy-Ado (9) were also poor substrates. Compounds with 2'-modifications were poor inhibitors unless they also had a second substitution at the 2-position.

3' A trans-3'-hydroxyl group was nearly a requirement for substrate and inhibitor recognition, although 9-[β -D-xylofuranosyl]-adenine (23), which has a cis-3'-hydroxyl group, was a poor substrate with 1 nmol/mg-min of activity. Modifications at this position may be limited by steric hindrance.

4'-O The 4'-oxygen was the most flexible of the substitutions to the ribose moiety. Carbocyclic-Ado (3) was a good substrate for both human and *M. tuberculosis* Ado kinases. The selectivity for carbocyclic-Ado analogs can be improved with the addition of an exocyclic substitution at the 2-position of the adenine base.

5' Substitutions to the 5-position were promising in terms of substrate activity and antitubercular activity. The best substrates in this category were 9-[α -L-lyxofuranosyl]-adenine (34) and 9-[α -L-lyxofuranosyl]-2-fluoroadenine (35), both maintained about 3% of the activity of Ado. Compounds with an additional 5'-methyl group in the *allo* and *talo* conformations (36–39) revealed that the *allo*- conformations resulted in better substrates and inhibitors of Ado kinase. Although it has yet to be confirmed, 5'-amino-5'-deoxy-Ado (32) appeared to be a substrate for this enzyme. This compound was also the best inhibitor of all of the substitutions to the ribose moiety.

Glycosidic bond position The preferred position for the *N*-glycosidic bond was at the 9-position of Ado. However, activity was measured with 3-[β -D-ribofuranosyl]-adenine (47) as well. Ado kinase did not recognize compounds as substrates when the bond was moved to the 7 or 8-positions on Ado (45, 46, and 48).
