2006

Adenosine kinase from Mycobacterium tuberculosis.

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ADENOSINE KINASE FROM *MYCOBACTERIUM TUBERCULOSIS*

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2006

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MARY C. LONG

ABSTRACT

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* has allowed this old pathogen to resurface as a major threat to world health. New drugs with novel mechanisms are needed in order to treat these increasingly drug-resistant strains. Nucleoside analogs are attractive for development as antimycobacterial drugs since these compounds are likely to have a mechanism of action that differs from existing antimycobacterial therapies. In order to develop purine nucleosides as antimycobacterial drugs, it is necessary to study the purine salvage enzymes that will be involved in their mechanism of action. Adenosine kinase (Ado kinase) was identified as an enzyme that is necessary for the mechanism of action of 2-methyl-Ado, a purine analog with selective antitubercular activity. Aside from measurements of its activity, little else was known about Ado kinase from *M. tuberculosis.* This work describes the purification and characterization of Ado kinase as well as identification and cloning of the *adoK* gene.

Ado kinase was purified to homogeneity from *M. tuberculosis* extracts in a four-step purification process and protein sequencing permitted the identification of Rv2202c as the gene which codes for Ado kinase. Ado kinase from *M. tuberculosis* is unique from other known Ado kinases in terms of primary structure, quaternary structure, substrate

preferences, and stimulation by potassium. Phylogenetic analysis demonstrated that Ado kinase from *M. tuberculosis* is more closely related to ribokinase than to other Ado kinases. Cloning Rv2202c into Ado kinase-deficient mycobacterium strains restored Ado kinase activity and sensitivity to methyl-Ado, and confirmed that the gene coded for Ado kinase. Structure-activity studies were performed in order to better understand the topography of the active site and advance drug development. One-hundred fifty adenosine analogs were evaluated as substrates and inhibitors of *M. tuberculosis* Ado kinase and a subset of these were selected for further evaluation in the human homolog. This work has identified differences in the substrate specificity between *M. tuberculosis* and human Ado kinases which may be exploited for the development of purine nucleosides as antitubercular drugs

DEDICATION

To my family which imparted me with a passion for science, nurtured my curious nature, and provided inexhaustible support, and to the memory of my father, E. Duane Long, whose innovative nature was inspiring and whose support will never be forgotten. He believed that I could do anything.

ACKNOWLEDGEMENTS

I want to acknowledge Dr. William B. Parker, my graduate advisor, who provided expert advice throughout this research project. He struck a fine balance between guiding the research and enabling me to work independently, and thereby grow as an investigator. He embodies both high standards for research excellence and a respectful demeanor, qualities which made for an ideal mentor.

I owe debts of gratitude to Dr. Mahmoud el Kouni and Dr. Vincent Escuyer, who also played large roles in advising me throughout my graduate work. I learned something new each time I spoke with Dr. el Kouni, who contributed his expertise in the field of nucleic acid research and wisdom imparted by decades of experience. I also worked closely with Dr. Escuyer, whose passion for bacteriology and molecular biology were inspiring for me. I am grateful for your staunch support and the lessons that I have learned from both of you.

Finally, I would like to acknowledge my co-workers who helped out in various ways each day. Foremost among these are Sue Shaddix and Paula Allan who taught me techniques and shared their friendship, and Hitoshi Someya who was a stalwart companion with whom I shared many scientific, philosophical, and personal discussions. It has been a pleasure working with each of you.

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LIST OF ABBREVIATIONS

acyclo-Ado	9-(2-hydroxyethoxymethyl)-adenine
ACV	Acyclovir
Ade	Adenine
Ado	Adenosine
adoK	The gene that codes for Ado kinase, also known as *CbhK* and Rv2202c
APRTase	Adenine phosphoribosyl transferase
araA	9-[β-D-arabinofuranosyl]-adenine
ATP	Adenosine triphosphate
AZT	Zidovudine, 3'-azido-3'-deoxythymidine
cAMP	Adenosine-3',5'-cyclic monophosphate
COPD	Chronic obstructive pulmonary disease
CTP	Cytidine triphosphate
BCG	Bacillus of Calmette and Guérin
dATP	Deoxyadenosine triphosphate
dCF	2'-Deoxycoformycin
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethylsulfoxide
DOTS	Directly observed therapy- shortcourse

DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
Formycin A	8-aza-9-deaza-adenosine
GTP	Guanosine triphosphate
HGPRTase	Hypoxanthine-guanine phosphoribosyl transferase
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
Ino	Inosine
LTBI	Latent tuberculosis infection
MDR-TB	Multidrug-reisitant tuberculosis
methyl-Ado	2-methyl-adenosine
MIC	Minimum inhibitory concentration
OADC	Oleic acid, albumin, dextrose, and catalase
P_i	Inorganic phosphate
PNP	Purine nucleoside phosphorylase
Ribavirin	1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
TAACF	Tuberculosis antimicrobial acquisition and coordinating facility
WHO	World health organization
XDR-TB	Extensively drug-resistant tuberculosis

CHAPTER I

INTRODUCTION

Tuberculosis Presents a Persistent Health Problem

Worldwide, tuberculosis is a growing health concern, infecting one-third of the worlds' population and claiming over two million lives annually [1-3]. The etiological agent for tuberculosis is *Mycobacterium tuberculosis*, identified for the first time in 1882 as an acid-fast bacillus by Robert Koch [4]. At the time that it was identified, *M. tuberculosis* claimed the lives of one in seven people; however, the incidence of infection decreased throughout most of the twentieth century due to the availability of effective antibiotics and the Bacillus of Calmette and Guérin (BCG) vaccine [4, 5]. The downward trend in tuberculosis incidence and mortality continued until it reached a nadir in 1984. Between 1985 and 1992, tuberculosis began to re-emerge as a global health concern, with a 20% increase in newly reported cases [5]. In 1993, the increased incidence of tuberculosis prompted the World Health Organization (WHO) to declare tuberculosis as a global health emergency. The concurrent timing of the human immunodeficiency virus (HIV) epidemic and the emergence of multi-drug-resistant strains of *M. tuberculosis* (MDR-TB) made the greatest contribution to the re-emergence of tuberculosis in the 1980's [5-7].

Consistent with the slow growth of *M. tuberculosis*, tuberculosis often takes years to run its course. The severity of tuberculosis symptoms covers a wide range, from asymptomatic carriage to severe cavitary disease. Among the symptoms of tuberculosis are malaise, weight loss, night sweats, cough, blood in sputum, chest pain, and breathlessness. A diagnosis of tuberculosis requires a chest x-ray showing cavitary lesions, conversion to a positive PPD skin test for unvaccinated individuals, and culture of the organism from sputum smears or other fluids. Extrapulmonary tuberculosis occurs occasionally in immunocompetent individuals, and is common in HIV-infected patients. Even though *M. tuberculosis* infects one-third of the world's population, only about 10% of infected individuals will develop active, or cavitary, tuberculosis infection in their lifetime. However, the *annual* risk for an HIV-infected individual is at least 8% [6, 8, 9]. Most infected individuals will either clear the infection or develop latent tuberculosis infection (LTBI). A small percentage of the population with LTBI will develop reactivation tuberculosis, a condition where severe tuberculosis disease occurs after a period of latency - usually years after initial infection. Risk factors for reactivation tuberculosis, increased age and HIV infection, are associated with a weakened immune system.

HIV infection is a major risk factor for tuberculosis and co-infection worsens the course of both diseases, resulting in an increase in morbidity and mortality [6, 7]. Infection with HIV created a large reservoir of people with weakened immune systems who were highly susceptible to primary tuberculosis infection and reactivation disease [6]. In sub-Saharan Africa, where HIV prevalence is high, 31% of adult tuberculosis cases were attributable to HIV [6]. Worldwide, the relationship between tuberculosis and HIV is

such that a diagnosis of tuberculosis is sufficient to warrant testing for HIV, indeed the finding of tuberculosis is often the sentinel event that leads to the diagnosis of HIV [1]. The severity of tuberculosis is also much greater among HIV-infected individuals, who are more likely to develop the active form of the disease. In many developing countries, the case fatality rate from tuberculosis in HIV-infected patients can be over 50% [6]. Drug therapy is also complicated for HIV-infected individuals due to an increased risk of drug-resistance, relapse, immune reconstitution syndrome, and overlapping toxicity profiles with antiretroviral drugs [1, 10]. Several commonly used HIV antiviral drugs and antitubercular drugs are potent inducers of cytochrome P450 drug-metabolizing enzymes. Patients who are being treated for both diseases can have inadequate dosing due to the induction of these drug-metabolizing enzymes.

While HIV-tuberculosis co-infection has drastically increased the incidence of tuberculosis worldwide, MDR-TB is making the disease much more difficult to treat. MDR-TB is defined as organisms which are resistant to at least isoniazid and rifampin and requires the use of second-line drugs that are less effective, costlier, and more toxic than isoniazid and rifampin-based regimens. The greatest risk factors for development of drug-resistant tuberculosis is a history of treatment for tuberculosis or HIV-tuberculosis coinfection [10, 11]. Any time there is low patient compliance, interruption of the drug supply, or premature discontinuation of therapy, there is a high risk of developing drug-resistant strains of tuberculosis. The largest example of a systematic breakdown of tuberculosis treatment occurred in Russian prisons, where many cases of MDR-TB originated

[12]. Eastern European countries continue to pay the price for this breakdown by sporting the greatest prevalence of MDR -TB in the world [7, 13].

Recently, WHO defined a new category of extensively drug-resistant (XDR-TB) [11]. These strains are resistant to isoniazid, rifampin, and at least three of the six classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinilones, thioamides, cycloserine, and *p*-aminosalicylic acid). Furthermore, patients with XDR-TB were at least 54% more likely to die during treatment than patients with MDR-TB [11]. MDR-TB and XDR-TB strains have been identified worldwide and they are predicted to comprise an increasing percentage of new tuberculosis cases.

The phenomenon of antibiotic-resistant strains of *M. tuberculosis* began to appear almost as soon as a new drug was introduced [10]. Since *M. tuberculosis* becomes rapidly resistant to a single drug, multidrug therapy was instituted in order increase the chances that an individual will be cured [10]. The basis for drug-resistance in *M. tuberculosis* is randomly-acquired, chromosomally-borne mutations [7, 10]. Drug resistance genes are not found on plasmids as they are in many other bacteria, probably because the drugs used to treat *M. tuberculosis* are not wide-spectrum antibiotics. Since mutations that impart resistance do not require drug exposure and these mutations are not linked, the probability of acquiring resistance to multiple drugs in this manner is quite low (i.e. resistance to isoniazid or rifampin is roughly 1 in 10^8 bacteria, whereas resistance to both occurs in 1 in 10^{16} bacteria). However, in the case of inadequate therapy, such as monotherapy, er-

ratic drug ingestion, suboptimal dosage, or poor drug absorption, *M. tuberculosis* may rapidly become resistant to multiple drugs [7, 10].

The Current Status of Tuberculosis Control

Tuberculosis is a treatable and preventable disease. Current antitubercular drugs are good at curing susceptible strains of *M. tuberculosis*, and proper hygiene practices coupled with treatment of infected individuals can prevent new infection. Worldwide, the greatest burden of tuberculosis falls on developing countries where the public health infrastructure is weak, there is little accessibility to good healthcare and quality medicines, and the populace cannot afford the cost of the necessary medications. The risk of treatment drop-out in these countries is very high. In order to address these issues, the WHO instituted the directly observed therapy, short-course (DOTS) program for tuberculosis detection and treatment. This program requires a strong commitment from the sponsoring government to provide a strong public health infrastructure. DOTS provides tools for tuberculosis diagnosis, standardized short-course therapy, a safe supply of high-quality drugs, directly observed treatment, and monitoring of program performance. In countries where DOTS has been successfully implemented, standard short-course regimens can cure more than 80% of drug-susceptible tuberculosis cases [2, 14].

In addition to treatment of existing cases, prevention of new infection is another goal for improved global health. A person with an active case of tuberculosis infects 10 to 15 people per year if they are not receiving treatment [15]. Ideally, an effective vaccine would provide protection against primary infection, prevent reactivation in persistently

infected individuals, and boost the body's immune response to active tuberculosis infection. The BCG vaccine that is currently in use protects against disseminated and meningeal forms of tuberculosis in young children. Unfortunately, BCG does not protect against pulmonary tuberculosis and thus, it has had limited impact on the global burden of tuberculosis [8, 14, 16]. Furthermore, the BCG vaccine complicates tuberculosis testing, rendering the tuberculin skin test ineffective for tuberculosis surveillance in countries where it is in use. One reason cited for the failure of the BCG vaccine is that it fails to elicit a robust immune response; therefore, several groups are attempting to improve the body's CD8 T-cell response to the BCG vaccine [8, 9, 16]. One group is including a gene from *Listeria monocytogenes* that elicits a strong CD8 response[8]. Another group is trying a prime-boost vaccination strategy where individuals initially receive the BCG vaccine and are later boosted with a different TB vaccine in order to elicit a stronger immune response [8]. Since development of a new tuberculosis vaccine is predicted to take 25 years or more, discovery of new antitubercular drugs is essential [14].

The most important drugs in the anti-tuberculosis arsenal are the first-line drugs: ethambutol, isoniazid, rifampin, pyrazinamide, and streptomycin (Table 1). The therapeutic regimen for patients who have not been previously treated consists of two months of isoniazid, rifampicin, pyrazinamide, and ethambutol followed by a four month continuation phase with isoniazid and rifampicin [1, 10, 17]. In the case of infection with drug-resistant strains of *M. tuberculosis*, treatment regimens can last well over a year and involve more drugs [1, 10, 11]. Strains of tuberculosis that are resistant to first-line drugs are increasingly prevalent, making health care workers more dependent on second-line

drugs such as fluoroquinilones (ofloxacin and ciprofloxacin), *p*-aminosalicylic acid, ethionamide, cycloserine, injectable aminoglycosides (amikacin, kanamycin, and capreomycin). Some other second-line drugs that are less-commonly used include amithiozone, clofazimine, amoxicillin-clavulinate, clarithromycin, azithromycin, and newer rifamycins. However, most of these drugs have not been FDA-approved for treatment of tuberculosis due to only modest antitubercular activity, unproven clinical efficacy, cross-resistance with first-line drugs, or very high minimum inhibitory concentration (MIC) values relative to the maximum achievable serum concentrations [10]. No new antitubercular drugs have been introduced since 1962, making it increasingly difficult to treat emerging strains of MDR-TB and XDR-TB [10, 18-20]. In order to avoid repeating mistakes of the past, it is imperative to avoid complacency when it comes to drug development. *M. tuberculosis* has proven to be adept at developing resistance to new pharmacological therapies and several new drugs will be needed to continue to treat MDR-TB and XDR-TB.

Table 1.

Front-line antitubercular drugs

Drug	Site of Action	Toxicity
Ethambutol	Inhibits arabinogalactan synthesis and mycolic acid incorporation into the mycobacterial cell wall	visual disturbances, rash, and fever
Isoniazid	Inhibits mycolic acid synthesis by the fatty acid synthesis II pathway - requires activation by catalase-peroxidase	neurotoxic effects (may be alleviated by administration of pyridoxine), hepatotoxic; induces cytochrome p450 enzymes
Pyrazinamide	bactericidal at acidic pH; may inhibit electron transport	hepatotoxicity, hyperuricemia due to inhibition of urate excretion, nongouty ployarthralgia
Rifampin	Inhibits bacterial DNA-dependent RNA polymerase	rash, thrombocytopenia, nephritis, liver dysfunction; strong inducer of cytochrome p450 enzymes
Streptomycin	aminoglycoside - inhibits protein synthesis by binding the 30S ribosomal subunit	ototoxicity, nephrotoxicity,neuromuscular blockade, skin reactions

Tuberculosis Drug Development Pipeline

In response to the WHO's call for new drugs to treat tuberculosis, several national and multinational organizations were formed in order to promote antitubercular drug development. The Global Alliance for tuberculosis drug development (Global Alliance) is a not-for-profit venture, whose goal is "to accelerate the discovery and development of new drugs to fight tuberculosis", has outlined three goals for novel antitubercular drugs [14]. New treatments should shorten the duration of treatment, improve the treatment of MDR-TB, and/or provide a more effective treatment LTBI, which is essential for eliminating tuberculosis. In order to achieve the goal of improving treatment for MDR-TB, new drugs should avoid the problem of cross-resistance with existing antitubercular drugs. Therefore, new drugs should have a mechanism of action that differs from existing therapies.

In the United States, the National Institute of Allergy and Infectious Disease established the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) in order to accelerate the testing and development of new antitubercular drugs [21]. The TAACF provides an initial in vitro screen against *M. tuberculosis* H37Rv. Promising compounds will advance through several stages including determination of minimum inhibitory concentration (MIC) in drug susceptible and drug-resistant strains of *M. tuberculosis*, cytotoxicity in Vero cells, efficacy in infected macrophage, and in vivo testing in a mouse model. The TAACF provides rapid, uniform testing of potential antitubercular compounds from both industry and academia.

The current status of anti-tuberculosis drug research holds promise for fulfilling some of the goals of the global alliance. As of March of 2006, four tuberculosis drug candidates diarylquinilone (R207910, Johnson and Johnson), Moxifloxacin (Bayer), Gatifloxacin (European commission, WHO), and pyrrole LL-3858 (Lupin Limited) were undergoing clinical evaluation [18, 20, 22-24]. Among the compounds that have been the most promising in advanced stages of preclinical testing are the nitroimidazole, PA-824 (Chiron, TB Alliance) and the ethambutol analog, SQ109 (Sequella). Multidrug therapy is standard for tuberculosis treatment. Therefore, new drugs need to be tested both alone and in combination with existing antitubercular drugs in order to optimize treatment while minimizing toxicity profiles. The threat of MDR-TB and XDR-TB strains of *M tuberculosis* becoming resistant to new therapies will maintain pressure to continue to develop new antitubercular drugs.

The Purine Salvage Pathway is a Target for Development of Antitubercular Nucleosides

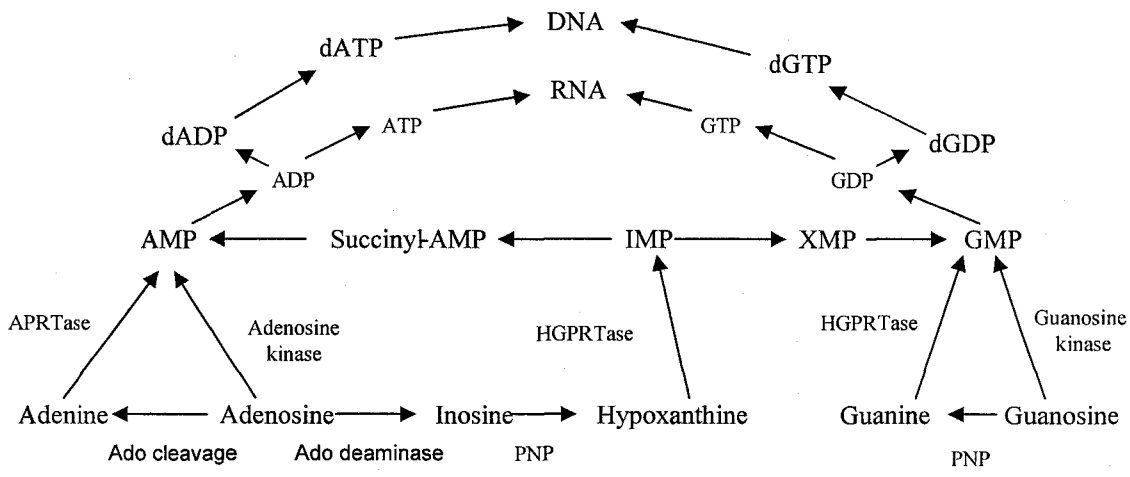
Many *Mycobacterium spp* possess enzymes required for *de novo* synthesis of purine and pyrimidine nucleotides [25-30]. Since *de novo* synthesis is metabolically expensive, many *Mycobacterium spp* also possess enzymes that salvage preformed purines and pyrimidines (Figure 1) [25-28, 31]. Although *M. tuberculosis* was not investigated in those studies, it is likely to contain many of the same purine salvage enzymes. Indeed, recent work has verified the presence of several purine salvage enzymes in *M. tuberculosis* including Ado deaminase, purine nucleoside phosphorylase, Ado cleavage, Ado kinase, and nucleoside diphosphate kinase [32-36]. Purine nucleotides, nucleosides and

nucleobases are in constant flux. Anabolic reactions convert nucleobases and nucleosides to mono-, di-, and triphosphate nucleotides. Nucleotides, particularly the triphosphates, such as adenosine triphosphate (ATP), are central to cellular energy transduction, DNA replication, RNA synthesis, and play an important role in metabolism by serving as bio-synthetic intermediates and cofactors. [37, 38]. Purine nucleosides and nucleobases are often products of RNA degradation and other catabolic reactions. Purine salvage enzymes such as phosphoribosyltransferases and nucleoside kinases permit the recycling of purine nucleosides and nucleobases back into the nucleotide pool. Salvaging the preformed bases and nucleosides permits organisms to save the metabolic energy that is required for *de novo* synthesis.

The purine salvage pathway in *M. tuberculosis* provides an attractive target for development of new antitubercular drugs [39]. Pathogenic *Mycobacterium spp* are intra-cellular pathogens, residing within unfused lysosomal compartments in infected macro-phages. In this environment, *Mycobacterium spp* have access to many cellular nutrients and molecules; among these are cellular nucleotides, nucleosides and nucleobases [25, 28, 40]. Nucleosides and nucleobases are taken up by the bacterium, but nucleotides are not taken up because the molecules carry a negative charge at physiological pH. Nucleo-tides need to be converted to nucleosides or nucleobases in order to be taken up by the bacterium. *M. leprae* has developed a method for converting host nucleotides to nucleo-bases using phosphatases that are acquired from its host cell and adhered to the surface of the bacillus; therefore, it is able to salvage exogenously supplied nucleotides as well [27,

41]. Although this pathway has not been studied in *M. tuberculosis*, it may have a similar mechanism for salvaging host-provided nucleotides.

Figure 1.



Purine salvage enzymes in *M. tuberculosis*. Abbreviations are APRTase, adenine phosphoribosyltransferase; HGPRTase, hypoxanthine-guanine phosphoribosyl transferase; PNP, purine nucleoside phosphorylase.

It may be possible to exploit the differences in the human and *M. tuberculosis* purine salvage pathways in order to develop nucleoside analogs that are selectively toxic to *M. tuberculosis*. Indeed, some 6-arylpurines have demonstrated potent antitubercular activity with good toxicity profiles *in vivo* [24, 42-47]. Little is known about the mechanism of action of these purine analogs; however, they probably inhibit an enzyme which utilizes purines. The use of nucleoside analogs is a strategy that has proven effective in developing anticancer, antiviral, and immunosuppressive drugs [38, 48, 49]. Although they have demonstrated antibacterial efficacy in the past, nucleoside analogs have not been aggressively developed for this purpose, presumptively because there were already a large number of antibiotics available [48]. Nucleoside analogs are considered antimetabolites because they resemble naturally occurring nucleosides. Although they have many different mechanisms of action, nucleoside analogs share the ability to be readily taken up by cells in an inactive form and are often converted to toxic metabolites by at least one enzyme before inhibiting a final target, hence they are usually considered to be prodrugs.

The same nucleic acids are the universal building blocks for RNA and DNA for all living things. Therefore, the challenge for designing a nucleoside analog that is selectively toxic to *M. tuberculosis* relies on identifying differences between the host and pathogen nucleoside metabolizing enzymes in a manner similar to antiviral nucleoside analogs. Antiviral nucleosides can exploit differences between host and virus proteins in order to target only virus-infected cells and not host cells. Viruses possess several enzymes involved in DNA or RNA metabolism which are very different from those found in the human host. Nucleoside analogs may be preferentially activated by viral enzymes

as in the case of acyclovir (ACV), which is a much better substrate for herpesvirus thymidine kinase than human nucleoside kinases. As a result, ACV triphosphates (acyclo-guanosine triphosphate, acyclo-GTP) are formed at a much higher rate in virus-infected cells. Acyclo-GTP acts as a chain terminator for nascent DNA strands and it selectively inhibits viral DNA polymerase. Another common mechanism is that nucleosides may be metabolized by host salvage enzymes but selectively inhibit viral targets. 3'-Azido-3'-deoxythymidine (Azidothymidine, Zidovudine or AZT) is an antiretroviral thymidine analog that has been important for the treatment of HIV infection. AZT is phosphorylated by host kinases to its triphosphate form, which is a potent inhibitor of HIV reverse transcriptase and it is also a chain-terminator when incorporated into a growing strand of DNA. However, it does not efficiently inhibit human DNA polymerase and is therefore selective against HIV.

Anticancer nucleosides differ in their basis for selectivity from antiviral nucleosides. Unlike most somatic cells, cancer cells are rapidly dividing and have a need for nucleic acids to support DNA replication. Most nucleoside analogs are phosphorylated to their respective nucleotides and inhibit a critical intracellular process such as nucleic acid synthesis, DNA polymerase, or they may be (mis)incorporated into DNA, where they result in chain termination or increase the numbers of mispaired nucleotides. Since the DNA proofreading and repair processes are often not as efficient in cancer cells, the accumulation of errors often results in cell death. Since cancer cells are of somatic origin there is a very narrow therapeutic window where cancer cell kill occurs and somatic cells survive, resulting in unwanted toxicity to proliferating somatic cells.

M. tuberculosis is a very slow-growing pathogen; therefore, it is unlikely that antitubercular drugs can exploit differences in cellular proliferation rates like anticancer nucleosides. Development of antitubercular nucleoside analogs is likely to more closely resemble antiviral drug development, where differences between host and pathogen proteins will provide the basis for selectivity. The intracellular compartmentalization of *M. tuberculosis* provides an additional challenge to drug development as nucleoside analogs will have to be taken up by infected macrophages and transported intact to the bacterium. Like viral pathogens, *M. tuberculosis* contains purine salvage enzymes that may be used to convert a nucleoside analog to a compound with antitubercular activity. Some of the genes that code for purine salvage enzymes have been identified by homology with genes of known function [50]. However, many of the genes which code for purine salvage enzymes could not be identified by homology searches. This indicates that purine salvage enzymes in *Mycobacterium spp* differ significantly from known enzymes even though they are functionally the same. The enzymes of purine salvage in *M. tuberculosis* must be identified and studied in order to develop nucleoside analogs as antitubercular drugs. Specifically, the successful development of these compounds depends on identifying and exploiting differences in substrate specificity between human and *M. tuberculosis* enzymes which will permit the selective activation of nucleoside analog prodrugs in *M. tuberculosis*.

2-Methyl-Adenosine is a Lead Compound for Antitubercular Drug Development

Several nucleoside and nucleobase analogs demonstrated antitubercular activity in a TAACF screen of compounds submitted by Southern Research Institute. Among these,

the adenosine (Ado) analog 2-methyl-adenosine (methyl-Ado, Figure 2) was identified as one of the most promising compounds, with a minimum inhibitory concentration (MIC) of 3 μg/ml and an IC_{50} of >1000 μg/ml in Vero cells and 80 μg/ml in CEM cells [51]. The TAACF further reported the following MIC values for methyl-Ado against strains of *M. tuberculosis* that are resistant to front-line drugs. The *M. tuberculosis* Erdman strain plus strains which were resistant to rifampicin, ethambutol, and kanamycin had MIC values of <1.56 μg/ml, whereas a isoniazid-resistant strain had an MIC of 3.13 μg/ml and a ciprofloxacin-resistant strain had an MIC of 6.25 μg/ml [51]. Furthermore, methyl-Ado was tested in a mouse model at 25 mg/kg per day for up to 45 days with no toxicity and no appearance of abnormal tissue [51]. Methyl-Ado proved to be slightly effective in the mouse model of infection with the noteworthy side-effect of methyl-Ado inducing sleep in the mice for several hours following administration of the drug (unpublished TAACF result). The activity of methyl-Ado provided proof-of-principle that nucleoside analogs can be developed as antitubercular drugs.

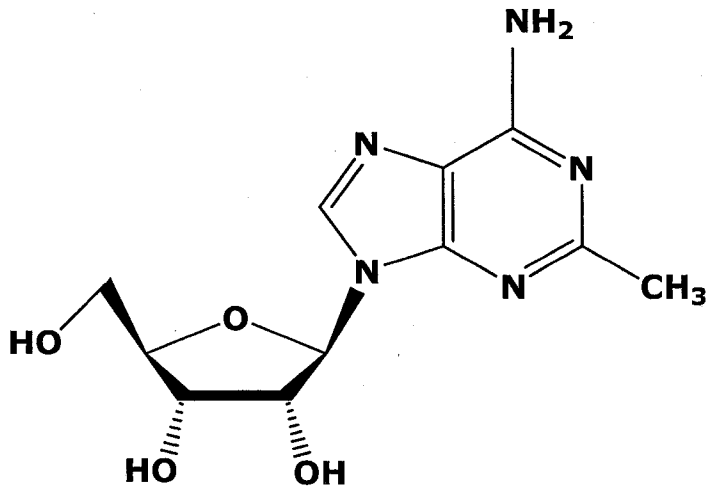
Due to its selective antitubercular activity, efforts were undertaken to understand the mechanism of action of methyl-Ado. Studies of intracellular metabolites were performed with cultures of *M. tuberculosis* H37Ra incubated with 10μM methyl-Ado. After 24 hours, 89% of the methyl-Ado had been converted to methyl-adenine and was found in culture supernatant and the remaining 11% had been converted to intracellular metabolites [32]. Methyl-AMP comprised 99% of the acid-soluble intracellular metabolites, with very little methyl-ADP or methyl-ATP formed [32]. After 24 hours, the acid-insoluble fraction incorporated only 0.02% of the metabolites of methyl-Ado. Furthermore, incuba-

tion with methyl-Ado had no effect on intracellular ATP concentrations. These results indicate that methyl-Ado does not inhibit de novo ATP synthesis and has very low rate of triphosphate formation and incorporation into bacterial RNA or DNA.

When CEM cells were similarly treated with methyl-Ado the predominant intracellular metabolites were methyl-AMP and methyl-ATP. These metabolites were formed at a rate of less than 0.5% of the rate of ATP formation [32]. However, in *M. tuberculosis*, the rate of conversion of methyl-Ado to methyl-AMP was 20% of the rate of conversion of Ado into intracellular metabolites [32]. The net result is at least a 40-fold difference in the rates of incorporation of methyl-Ado into intracellular metabolites. This difference in the rates of conversion may account for at least some of the selective activity of methyl-Ado.

Studies involving the effect of methyl-Ado on bacterial macromolecular processes confirmed that methyl-Ado does not affect RNA synthesis; rather it primarily inhibits protein synthesis, with delayed inhibition of DNA synthesis [51]. Since methyl-Ado closely resembles Ado, it is likely that metabolites of methyl-Ado inhibit an enzyme that normally utilizes adenosine nucleotides as a reactant or cofactor. It is difficult to predict the specific enzyme that is inhibited by methyl-Ado metabolites based on the large number of reactions that involve adenosine nucleotides.

Figure 2.



The chemical structure of 2-methyl-adenosine

Methyl-Ado-resistant strains of *M. smegmatis* were established previously by culturing *M. smegmatis* strain mc^2155 on Middlebrook 7H11 plates containing 10 μM methyl-Ado [52]. Resistant strains occurred at a frequency of 1 in 10^6 colony forming units. Studies of intracellular metabolites of one methyl-Ado resistant strain, SRICK1, revealed that methyl-AMP was not formed in this strain. Methyl-Ado may be converted to methyl-AMP by several pathways including cleavage to methyl-Ade, followed by phosphoribosylation to methyl-AMP; direct phosphorylation to methyl-AMP by Ado kinase; or deamination to methyl-inosine (Ino) by Ado deaminase, followed by conversion to methyl-AMP in a multi-enzymatic process involving purine nucleoside phosphorylase (PNP), hypoxanthine-guanine phosphoribosyl transferase, adenylosuccinate

synthase, and adenylosuccinate lyase. It was previously demonstrated that methyl-Ado was not a substrate for Ado deaminase or PNP from *M. smegmatis* [52]. Protein extracts of SRICK1 were deficient in Ado kinase and Ado cleavage activities [32].

Adenosine Kinase

Ado kinase (EC 2.7.1.20) phosphorylates Ado to AMP by transferring the γ-phosphate of ATP to Ado in a magnesium-dependent manner (Fig 3). Ado kinase is found in most types of organisms and has been purified from numerous mammalian, plant, and parasitic sources [53-67]. Ado kinase is constitutively expressed; however, Ado kinase activity may be upregulated under pathological conditions which result in an increase in intracellular Ado. Therefore, Ado kinase also can serve to regulate the intracellular Ado concentration. Ado can act as an endocrine or exocrine signaling molecule, affecting cardiovascular, nervous, respiratory, antiinflammatory, and immune system functions [63, 68-71]. These effects can be attributed to the interaction of Ado with Ado receptors, which are found throughout the body [69, 70, 72]. Indeed, it is possible that the sleep-inducing side-effect of methyl-Ado in mice was due to the interaction of methyl-Ado with these Ado receptors. Ado kinase plays a pharmacological role in activating the antiviral drug 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) and the immunosuppressive drug mizoribine [63, 73, 74]. In the past, the paradigm has been that bacteria do not have Ado kinase. However, Ado kinase activity has been measured in crude extracts from the intracellular bacteria *Chlamydia psittaci* and some *Mycobacterium spp* [25-27, 75]. Although activity has been measured in crude extracts, no work has been done to purify and characterize Ado kinase from these bacterial sources.

Ado kinase is a purine salvage enzyme and a member of the PfkB family of carbohydrate and nucleoside kinases [76, 77]. This family of structurally related enzymes includes ribokinase, fructokinase, ketohexokinase, inosine-guanosine kinase, 1-phosphofructokinase, and 6-phosphofructokinase among its members [76, 77]. PfkB family members are functionally related since they are all kinases that catalyze the transfer of the terminal phosphate from ATP or GTP to their respective substrates. Similarities in function necessitate some structural similarities. Crystal structures and sequence comparisons revealed that structural features such as an anion hole, an activating aspartic acid residue, and an NXXE motif are conserved in PfkB family members [62, 63, 76-81].

All of the Ado kinases that have been purified and characterized to date share several properties. Most are active monomers with a molecular mass between 34 and 40 kDa [55-57, 59-63, 66, 67, 82]. Ado and deoxyadenosine are the only natural nucleosides that are efficiently phosphorylated by Ado kinases. Inhibition of enzymatic activity has been reported at high concentrations of Ado, AMP and ATP [57, 59, 61, 66]. The reaction catalyzed by these proteins requires a divalent cation, usually magnesium; it is likely that Mg-ATP is the actual phosphate donor although Mg-GTP can also be efficiently used. There have been several reports of stimulation of Ado kinase activity in the presence of inorganic phosphate (P_i); however, this effect has not been routinely investigated for all Ado kinases [83-85]. While the amino acid sequences for many Ado kinases share greater than 50% homology, a few share less than 20% identity with other Ado kinases. Although low sequence homology may be sufficient to identify a gene as one that codes for a PfkB family protein, it is impossible to identify the exact function of the protein. In

the case of Ado kinase from *M. tuberculosis*, the gene that codes for Ado kinase could not be identified based on homology searches using known Ado kinase sequences. Since the Ado kinase gene could not be identified based on genetic analysis, traditional purification techniques were used in order to study *M. tuberculosis* Ado kinase.

In the current work, we have undertaken the task of studying Ado kinase from *M. tuberculosis* in an effort to understand the similarities and differences between it and its human homolog, particularly differences in substrate specificity that may impart selectivity to the metabolism of methyl-Ado. Initial studies involve purification of Ado kinase from crude extracts of *M. tuberculosis*, biochemical characterization of the enzyme, phylogenetic analysis, cloning, and preliminary crystallization of the protein. Further studies include a structure-activity relationship designed to probe the topography of the active site and identify modifications that may be made to adenosine that will benefit drug development.

The specific aims of this project are:

1. Purify and characterize Ado kinase from *M. tuberculosis* and identify the gene which codes for this protein.
2. Study the Ado-binding domain of Ado kinase by means of a structure-activity relationship in order to identify topographic features that may be exploited for the development of nucleoside analogs as antitubercular agents.

CHAPTER II

MATERIALS AND METHODS

Supplies and Cell Lines

Chemicals and reagents. Radiolabeled nucleosides including [2,8-^{3}H]Ado (30 Ci/mol), [8-^{3}H]guanosine (6.3 Ci/mmol), [2,8-^{3}H]inosine (14.6 Ci/mmol), [5-^{3}H]cytidine (25.3 Ci/mmol), [methyl-^{3}H]thymidine (60 Ci/mmol), [5-^{3}H]uridine (17.7 Ci/mmol), and [2,8-^{3}H]deoxyadenosine (13 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, California). Methyl-Ado was prepared at Southern Research Institute, Birmingham, Alabama [52], and radiolabeled by Moravek Biochemicals, Inc. to form [8-^{3}H]methyl-Ado (10.2 Ci/mmol).

Nucleoside analogs were obtained from several different sources. Ado **(1)**, 9-[β-D-ribofuranosyl]-purine, 6-chloro-purine riboside, 6-oxy-purine riboside, 6-mercapto-purine riboside, 6-methoxy-purine riboside, 8-bromo-Ado, 8-aza-9-deaza-Ado, 2'-deoxy-Ado, 2'-O-methyl-Ado, and 9-[β-D-arabinofuranosyl]-adenine were purchased from Sigma-Aldrich (St. Louis, MO). 6-Bromo-purine riboside, 6-iodo-purine riboside, 8-azido-Ado, 6-nitrobenzyl-mercapto-purine riboside, 6-benzyl-mercapto-purine riboside and 9-deaza-Ado, and 9-[β-L-ribofuranosyl]-adenine were kindly provided by Dr. Mahmoud el Kouni (University of Alabama at Birmingham, Birmingham, Al.) [86]. 2-Fluoro-

3-deaza-Ado was a gift from Dr. Alan C. Sartorelli (Yale University, New Haven, Ct.) [87]. The National Institutes of Health (Bethesda, Md.) provided 2-β-D-ribofuranosyl-thiazole-4-carboxamide, 2-β-D-ribofuranosylselenazole-4-carboxamide, and 2'-deoxy-coformycin. The National Cancer Institute (Bethesda, Md.) provided 7-deaza-7-carbox-amido-Ado. 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide was purchased from ICN Pharmaceuticals (Costa Mesa, Ca.). All other compounds were provided by the chemical repository at Southern Research Institute (Birmingham, Alabama). Each compound was solubilized in water or dimethylsulfoxide (DMSO) as necessary.

Bradford dye reagent, 12% SDS-PAGE mini gels, low molecular weight standards, and silver stain reagents were purchased from Bio-Rad laboratories (Hercules, California). The pVV16 shuttle vector was a gift from Dr. Varalakshmi Vissa (Colorado State University). Oligonucleotide primers were purchased from Operon Technologies (Alameda, California). Dynazyme EXT, was purchased from Finnzymes (Espoo, Finland). The Qiagen miniprep kit (Valencia, California) was used to purify plasmid DNA. High-pressure liquid chromatography (HPLC)-grade ammonium dihydrogen phosphate (monobasic ammonium phosphate) was purchased from Fisher Scientific.

Cell lines and culture conditions. *M. tuberculosis* strain H37Ra (ATCC 25177) and *M. smegmatis* strain mc^2155 (ATCC 700084) were cultured in Middlebrook 7H9 media supplemented with oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween-80. Previously-derived Ado kinase-deficient strains of *Mycobacteria*, SRICK1 and SRI101, were used for activity complementation studies [52]. SRICK1 is an Ado kinase-deficient

spontaneous mutant obtained by growing *M. tuberculosis* H37Ra on Middlebrook 7H10 containing 10 μg/ml methyl-Ado. Mutants occurred with a frequency of about 1 in 10^6 cells (unpublished results). SRI101 was created by transposon mutagenesis of *M. smegmatis* mc^2155 [52]. AK-deficient strains were grown in Middlebrook 7H9 media containing OADC and 0.05% Tween-80, supplemented when necessary with 50 μg/ml of hygromycin. *Escherichia coli* strain DH5α was cultured in Luria-Bertani media supplemented when necessary with 50 μg/ml of kanamycin. Bacteria were grown at 37°C under either stationary or shaking conditions, as required.

CCRF-CEM cells (ATCC CCL-119) were used as a source of native human Ado kinase. CCRF-CEM cells were cultured in RPMI 1640 media supplemented with L-glutamine, 10% fetal bovine serum, and 25 mM HEPES. Cultures were grown at 37°C with 5% CO_2.

Cloning and Expression

Cloning and expression of Ado kinase from M. tuberculosis. The *RV2202c* gene (Accession Q10391) was amplified by PCR from *M. tuberculosis* strain H37Rv chromosomal DNA using oligonucleotide primers 5'-GGACGGAGATCATATGACGATCGCGGTAAC-3' and 3'-ACGCCGAGCGACTAGACGTCGTGGTGCGAC-5', containing NdeI and PstI restriction sites respectively [50]. PCR product was digested with NdeI and PstI restriction enzymes and cloned into a pVV16 shuttle vector, which imparts hygromycin resistance. After propagation in *E. coli* strain DH5α, plasmid DNA was purified according to the Qiagen miniprep protocol. DNA sequencing confirmed that cloned *Rv2202c*

had no mutations. The final construct (pVV16/*Rv2202c*) was transformed by electroporation into AK-deficient *M. smegmatis* and *M. tuberculosis* strains, SRI101 and SRICK1 respectively, and transcription was driven by the *hsp60* promoter present in pVV16.

Rv2202c was also cloned into *E. coli* strain BL21 in order to overexpress protein for crystallization experiments. Rv2202c was amplified by PCR from the genomic DNA of the *M. tuberculosis* strain H37Rv using two primers for upstream and downstream sequences: 5'-GGACGGAGATCATATGACGATCGCGGTAACC-3' (upstream) and 5'-CCACACGGTGGAATTCCGCGTCTGCTCGGC-3' (downstream). The DNA was subcloned using the NdeI and EcoRI restriction sites that were present in the pET 28a vector (Novagen) to generate a recombinant vector containing a 5' sequence encoding a 20 amino acid N-terminal polyhistidine tag and a thrombin cleavage site [88]. The entire coding sequence was verified by DNA sequencing.

The resulting plasmid was transformed into competent cells of *E. coli* strain BL21*(DE3) and the transformed cells were selected on LB agar plates containing 50 μg/ml kanamycin. For protein expression, the *E. coli* cells containing the recombinant vector were inoculated and grown at 37°C for 6 hours in LB medium containing 50 μg/ml kanamycin. When the optical absorption of the culture reached 0.6-0.8, protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and cultures were grown at 17°C for an additional 40 hours.

Expression of recombinant human Ado kinase. The human Ado kinase clone 20-1 was generously provided by Dr. Jozef Spychala (UNC Chapel Hill, Chapel Hill, NC) [63, 89]. Briefly, *Escherichia coli* strain BL21 was transformed with a pET24b vector containing the human Ado kinase gene [63]. 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 IPTG, and expression was allowed to proceed for 4 hours.

Protein Purification

Preparation of crude protein extracts. Protein was extracted from CEM cells, *M. tuberculosis* strains, and *E. coli* strains as follows. Cells were pelleted by centrifugation, resuspended with an equal volume of phosphate-buffered saline, and rinsed twice with 50 mM Tris-HCl (pH 7.5) containing 5 mM ethylenediamine tetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride, 2.3 μg/ml leupeptin, and 1.1 μg/ml pepstatin, and resuspended in the same buffer. Resuspended *M. tuberculosis* cells were disrupted either by French press or with 0.1 mm glass beads using a Mini bead beater apparatus (Bio Spec Products, Inc.). *E. coli* strains and CEM cells were disrupted by sonication. Particulate matter was removed by centrifugation at 3000 x *g* for 20 min., followed by centrifugation at 40,000 x *g* for 1 hour at 4°C in a Beckman L-70 ultracentrifuge. Supernatant was filtered through a 0.2 μm filter, and dialyzed against 50 mM Tris-HCl (pH 7.5) with 10 mM NaCl, 1 mM dithiothreitol (DTT), and 20% glycerol.

Purification of M. tuberculosis Ado kinase. Aliquots of the crude protein extract were subject to stepwise ammonium sulfate precipitation using 40%, 60%, and 80% ammonium sulfate. Precipitated pellets were resuspended in 50 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 1 mM DTT (buffer A), and the resuspended protein was dialyzed against the same buffer. Ado kinase activity was found in the 40 – 60% ammonium sulfate fraction. Protein was subject to separation by anion exchange chromatography on a Hi Trap Q column where separation was achieved by running a linear salt gradient from 100 to 400 mM NaCl in buffer A. Fractions containing Ado kinase activity were pooled, dialyzed against buffer A, then concentrated to a final volume of 200 µl using a Centricon Plus-20 centrifugal filter device (10,000 Da. Cutoff, Amicon). Concentrated protein was loaded onto a Superose 12 column and subjected to gel filtration chromatography using an isocratic run with buffer A. Fractions containing Ado kinase activity were pooled and applied to a Mono Q anion exchange column where a linear salt gradient from 100 to 400 mM NaCl in buffer A was used to elute protein. Protein concentrations were determined at each step by the Bradford method using bovine serum albumin as a standard [90]. Purified protein was subject to sequencing on a Beckman model PI 2090E, using Edman degradation. Peptide sequencing was carried out by the Protein Analysis Shared Facility of the Cancer Center at UAB with support from the NIH (CA13148).

Protein characterization for *M. tuberculosis* Ado kinase were performed with partially purified AK that had been carried through the first three steps of purification and had been shown to be free of Ado deaminase and AMP kinase activity, as these enzymes can interfere with Ado kinase kinetics by depleting the substrate and product respec-

tively. All enzyme reactions were linear during the incubation period, and substrate conversions were maintained in the five-to-ten percent range.

Preparation of native human Ado kinase. A CEM cell extract was subjected to precipitation with 50% ammonium sulfate and dialyzed against 50 mM Tris (pH 7.5) containing 10 mM NaCl, 1 mM DTT, and 10% glycerol (buffer B). Ado kinase activity was found in the >50% ammonium sulfate fraction. Protein was subject to separation by anion exchange chromatography on a Hi Trap Q column followed by cation exchange chromatography on a Hi Trap SP column. At pH 7.5, human Ado kinase did not adhere to either resin; however, more than 50% of the total protein was removed in each step. Preparation of human Ado kinase removed 83% of the total protein and provided a five-fold purification, with 75% recovery of Ado kinase activity. The partially purified protein preparation was found to be free of AMP kinase, but some Ado deaminase activity remained; therefore, deoxycoformycin (dCF, a potent Ado deaminase inhibitor) was included in all assays involving the human enzyme.

Purification of recombinant human Ado kinase. 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 1M 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 (AD) activity remained; therefore, dCF was included in all assays involving the human enzyme.

Activity Assays and Enzyme Kinetics

Ado kinase filter disc assays for radiolabeled substrates. *M. tuberculosis* Ado kinase activity was followed throughout purification by a filter disc assay, where phosphorylation of [^{3}H]Ado was quantified by the amount of [^{3}H]AMP bound to a DE-81 cellulose disk following the reaction. Assay conditions consisted of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM ATP, 20 μM [^{3}H]Ado (10 μCi/ml), 0.01% bovine serum albumin (BSA) and 10 μM dCF, an inhibitor of Ado deaminase. KCl (10 mM) was added to the reaction mixture when indicated. The reaction was started by the addition of enzyme, incubated for the desired time at 37°C, and stopped by the addition of 10 μl of 0.1 M EDTA. Aliquots of 50 μl of reaction mixture were applied to DEAE cellulose disks, 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. Human AK was assayed as described above with the following variations: 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP, 2 μM [^{3}H]Ado (10 μCi/ml), 10 μM dCF, and 40 mM KCl.

The specific activity of the enzyme was calculated for each experiment. Known parameters were the concentration of Ado (μM), the mass of protein (μg), the duration of the reaction (time- min), and the volume of each sample taken (μl). Radioactive counts per minute (CPM) were determined for each sample and radioactivity totals (the amount of radioactivity that is involved with 100% conversion of Ado → AMP) were counted for each experiment.

1. The total number of pmol of Ado were determined for each experiment as follows:

$$\text{Vol } (\mu l) \times \frac{\text{conc Ado } (\mu mol)}{\text{Liter}} \times \frac{\text{liter}}{10^6\ \mu L} \times \frac{10^6\ \text{pmol}}{\mu mol} = \text{total pmol of Ado} \quad \text{(equation 1)}$$

For a 50 μl sample that had 20 μM Ado, the total amount of Ado would be 1000 pmol.

2. The conversion factor that will be used for all specific activity calculations is calculated as follows:

Total mass of Ado from equation 1 (pmol)/ CPM for the total radioactivity control (equation 2)

For example: 1000 pmol/ 35,000 CPM = 2.86×10^{-2} pmol/CPM.

3. For each experimental sample, CPM are converted to specific activity as follows:

$$\frac{\#\ \text{CPM}}{\text{protein mass (mg)} \times \text{time (min)}} \times \frac{\text{pmol (from equation 2)}}{\text{CPM}} = \frac{\#\ \text{pmol}}{\mu g\text{-min}} \equiv \frac{\text{nmol}}{\text{mg-min}}$$

(equation 3)

For a sample that contained 10^{-4} μg of protein, incubated for an hour, that yielded 1024 CPM, the specific activity would be:

[1024 CPM/(10^{-4} μg x 60 min)] x 2.86 x 10^{-2} pmol/CPM = 4881 nmol/mg-min.

Ado kinase activity assays for non-labeled nucleosides. Activity assays for *M. tuberculosis* AK consisted of 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 0.01% BSA, 10 μM dCF, and 100 μM of the appropriate test compound. Substrates for human AK were 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 dCF, and 100 μM of the appropriate test compound.

Assays were performed as previously described [89]. Briefly, 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 time-point 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.

The specific activity calculations for these samples was based on the assumption that none of the monophosphate that was created was converted to any other metabolite (the protein was already shown to be free of AMP kinase); therefore, compounds would exist only as the parent nucleoside or the monophosphate metabolite. Summing the area under the nucleoside and nucleoside monophosphate peaks should account for 100% of the nucleoside substrate.

1) The percent of the parent nucleoside that was phosphorylated was determined as follows:

   Nucleoside area + nucleoside monophosphate area = total area (equation 4)

2) Nucleoside monophosphate area/ total area (from equation 4) = % conversion (equation 5)

Other known parameters from the assay design are mass of protein (mg), sample volume (µl), and duration of the reaction (time - min). Since 100 µM of test compound was used in each assay, the mass of monophosphate formed could be calculated as follows:

3) $\text{Vol } (\mu l) \times \frac{\text{conc Ado } (\mu mol)}{\text{liter}} \times \frac{\text{liter}}{10^6\ \mu L} \times \frac{10^6\ \text{pmol}}{\mu mol} = \text{total pmol of Ado}$ (equation 6)

   Since the conversion factors cancel out, the calculation simplifies as follows:

   50 µl x 100 µM nucleoside = 5000 pmol nucleoside

4) The amount of monophosphate formed is calculated as a percentage of the total amount of parent nucleoside present:

mass of nucleoside (pmol - from equation 6) x percent conversion (from equation 5) = pmol of monophosphate formed. (equation 7)

5) The reaction velocity (specific activity) is calculated by dividing this conversion through by the mass of protein (μg) and reaction time as follows:

$$\frac{\text{pmol of monophosphate formed (from equation 7)}}{\text{protein mass (μg) x time (min)}} = \text{velocity (pmol/μg-min)}$$

(equation 8)

For example:
310 pmol/ ($3x10^{-4}$ μg * 40 min) = 25,800 pmol/μg-min ≡ 25,800 nmol/mg-min.

Ado deaminase activity assay. Reaction mixtures for Ado deaminase were the same as for Ado kinase, with the exclusion of ATP. Reactions were incubated in a 37°C water bath for one hour. At appropriate 0, 20, 40, and 60-min timepoints, 50 μl aliquots were withdrawn and the reaction was terminated by the addition of an equal volume of 1 M perchloric acid. Samples were neutralized to pH 7 and precipitated salts were removed by centrifugation. Reactants and products (Ado and Ino) were separated using reverse-phase HPLC (Hypersil C-18 column, Keystone Scientific) with a mobile phase comprised of 2.5% acetonitrile in 25 mM monobasic ammonium phosphate. Ino and Ado were detected as they eluted by monitoring their absorbance at 260 nm.

Determination of the purity of nucleoside analogs. The purity of each compound used in the structure-activity relationship was determined by reverse-phase chromatography

using a Hypersil C-18 column (Keystone Scientific) with a gradient of 5% to 50% acetonitrile in 25 mM monobasic ammonium phosphate.

AMP kinase activity assay. AMP kinase reaction mixtures were the same as described above for Ado kinase, with the substitution of 20 μM [2,8-^{3}H]AMP for Ado. Incubation and detection conditions were the same as for Ado kinase.

Assays for inhibition of Ado phosphorylation. 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 treated as described above for Ado kinase activity. 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%.

Enzyme kinetics. Michaelis-Menton parameters were determined from linear double-reciprocal plots of 1/velocity *vs* 1/concentration of the substrate. The best line was determined by linear regression of at least five data points (regression coefficient for each point was greater than 0.95), and the K_m and V_{max} values were determined from the inter-

cept of the x and y axes, respectively. Values are expressed as mean ± standard error of the mean for at least three determinations.

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.

Amino Acid Sequence Alignment and Phylogenetic Analysis.

The amino acid sequence from *M. tuberculosis* Ado kinase was aligned with sequences from related Ado kinases and ribokinases using ClustalW sequence alignment (European Bioinformatics Institute, www.ebi.ac.uk/ClustalW). Alignment of *M. tuberculosis* Ado kinase with other bacterial Ado kinases was performed using Multalin software (http://www.toulouse.inra.fr/multalin/multalin.html). Phylogenetic analysis was performed with Genebee software (www.genebee.msu.su/index.html).

Determination of Native Molecular Mass.

The buffer used in each of the native molecular weight determination experiments was the same as that used in the purification of *M. tuberculosis* Ado kinase (buffer A). The native molecular weight of the enzyme was estimated using size exclusion chromatography. A Superose 12 analytical grade column (Amersham Pharmacia Biotech, Piscataway, New Jersey) was calibrated using molecular weight standards ranging from 25

to 669 kDa, and a calibration curve was created based on the elution volumes of these standards. The formula $K_{av} = (V_e-V_o)/V_t-V_o$ was used to calculate the migration constants. Where the void volume (V_o) was measured by exclusion of blue dextran, and total column volume (V_t) was provided with the column specifications. A calibration curve was created by plotting K_{av} vs log MW and the native molecular weight of Ado kinase was extrapolated from this curve.

In a separate experiment, a PD2020A light scattering detector (Precision Detectors, Franklin, Ma.) was used in conjunction with size exclusion chromatography. As peaks eluted from the column, changes in the refractive index and light scattering properties were collected using an 810 nm laser with detectors at 15° and 90°. Light scattering data were analyzed on Discovery 32 software (Precision Detectors).

The native molecular weight of the enzyme was verified by sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation using a Beckman XLA ultracentrifuge. Sedimentation velocity was determined by spinning three protein concentrations at 59,000 RPM for 10 hours. Sedimentation velocity data was collected at 250 and 280 nm, then analyzed with Sedfit87 software (National Institutes of Health, Bethesda, Md). Sedimentation equilibrium data was obtained for two protein concentrations at 10,000, 12,500, 15,000, and 18,000 RPM. Data was collected at 250 and 280 nm, then analyzed using Beckman XL-A/XL-1 data analysis software, version 4.0 (Fullerton, Ca.).

Mass Spectrometry of Cloned Ado kinase

The subunit molecular mass of cloned Ado kinase was verified using mass spectrometry. A Macromass LCT electrospray mass spectrometer (Waters Corporation, Milford, MA.) was used to determine the subunit molecular mass of Ado kinase after separation by liquid chromatography. Mass spectrometry data was analyzed with Masslynx software (ver 3.5) using a maximum entropy algorithm.

Determination of Minimum Inhibitory Concentration

The MIC for methyl-Ado was evaluated in *M. tuberculosis* strains H37Ra, SRICK1, and SRICK1-pVV16/*Rv2202c* using a colorimetric microdilution broth assay as previously described (29). Dilutions of methyl-Ado, ethambutol, and DMSO were prepared in Middlebrook 7H9 media supplemented with 0.2% glycerol and OADC. For each concentration of methyl-Ado, ethambutol, and DMSO, 50 µl of the appropriate dilution of compound were added to triplicate wells. Two of the three wells were inoculated with about 5×10^4 cfu/well in 50 µl of Middlebrook 7H9 supplemented with OADC, and 50 µl of Middlebrook 7H9 supplemented with OADC were added to the third well, which served as a color control. For methyl-Ado and ethambutol, seven dilutions were used ranging from 50 to 0.5 µg/ml, while DMSO concentrations ranged from 0.5 to 0.05%. Viability controls and media control wells were included in each assay. Assay plates were incubated at 37°C. On the sixth day post-inoculation, 50 µl containing Alamar blue dye reagent diluted in 0.05%Tween-80 were added to each test well, and plates were incubated for an additional 18 hours. Reduction of Alamar blue dye was measured on an optical microtiter plate reader programmed to subtract the absorbance at 600 nm from that at

570 nm. The MIC was reported as the lowest concentration of drug with a differential absorbance of zero or less.

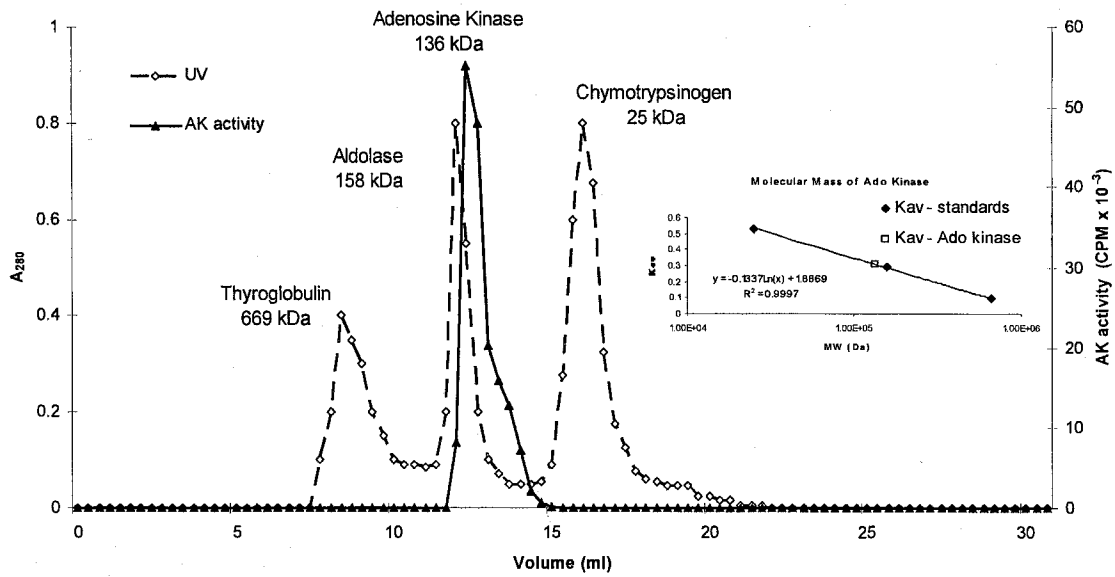
CHAPTER III

RESULTS

Purification of M. tuberculosis Ado kinase

Ammonium sulfate precipitation removed approximately 60% of contaminating proteins; however, it did not yield a large purification factor-fold due to the concurrent loss of Ado kinase activity (Table 2). The most efficient step in protein purification was anion exchange chromatography using the Hi Trap Q column, where Ado kinase eluted in fractions containing 250-320 mM NaCl. Ado kinase eluted from the Superose 12 gel filtration column in a single peak at 136 kDa (Figure 3). In the final purification step, enzyme eluted from the Mono Q anion exchange column at 260-320 mM NaCl. This procedure eliminated 99.99% of the protein and produced a 100-fold purification (Table 2). Only fractions containing maximum activity were carried over to the next step in order to minimize contamination by other proteins, which is reflected by the low (1%) percent yield. Enzyme preparations were stable when stored at -20°C in 20% glycerol for up to 6 months. The appearance of a single band at 35-kDa on a silver-stained SDS-PAGE gel (Figure 4) verified that Ado kinase was purified to apparent homogeneity. Purified protein maintained activity for > 2 years following purification when stored at -20° C in 40% glycerol.

Figure 3.



Elution profile of Ado kinase from a Superose 12 size exclusion column. The native molecular mass of Ado kinase was initially determined during purification using a Superose 12 size exclusion column. The column was run as described in the materials and methods section, 1-ml fractions were collected. Before purification, molecular mass standards were run and a calibration curve was created using thyroglobulin, aldolase, and chymotrypsinogen standards (669, 158, and 25 kDa, respectively). Molecular mass standards were detected as they eluted from the column by their absorbance at 280 nm and Ado kinase activity assays were performed on each 1-ml fraction. The K_{av} was calculated for each peak and plotted against the log MW in order to create a calibration curve. The native molecular mass of Ado kinase was determined to be 136 kDa based on this curve.

Table 2.

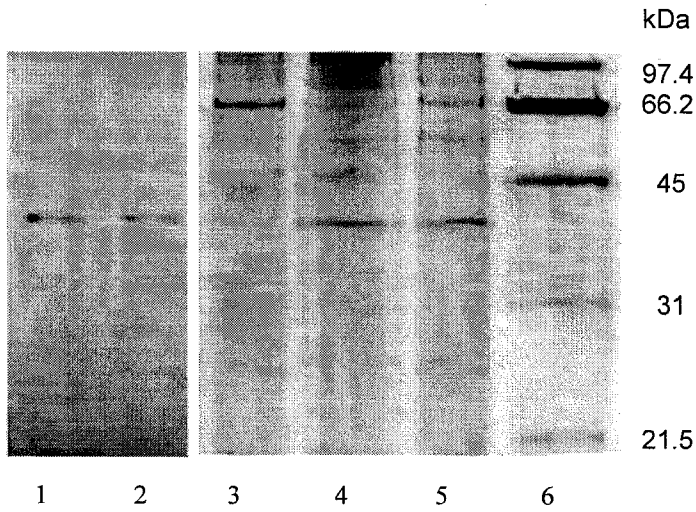
Purification table for Ado kinase from *M. tuberculosis*

Step	Volume	Total protein	Specific activity[a]	Purification factor[b]	Total activity[c]	Recovery[d]
	(ml)	(mg)	(nmol/mg-min)		(nmol/min)	(%)
M. tuberculosis crude	4	14,400	3.9	1	56,000	100
Ammonium sulfate	2.3	5,300	3.9	1	21,000	37
Hi Trap Q	0.2	92	53	14	4,900	9
Superose 12	1.6	14.1	115	30	1,600	3
Mono Q	0.3	1.4	390	100	550	1

a. Specific activity was calculated from Ado kinase activity assays as described in materials and methods.
b. Purification factor is calculated as the new specific activity/ specific activity in the crude extract.
c. Total activity is calculated as the specific activity x total protein.
d. Recovery is calculated by total activity/ total activity in the crude extract.

Note: From "Identification and Characterization of a Unique Adenosine kinase from Mycobacterium tuberculosis" by M.C. long, V. Escuyer, and W.B. Parker, 2003, *Journal of Bacteriology*, 185 (22), p. 6548-55. Copyright 2003 by ASM Press. Reprinted with permission.

Figure 4.



Molecular mass of Ado kinase purified from *M. tuberculosis*. The subunit molecular mass of purified Ado kinase was estimated based on a denaturing SDS-12% PAGE. Lanes 1 and 2 contain Mono Q fractions with Ado kinase activity from purification of the native enzyme. The mass of protein that was loaded onto the gel was not determined because of the limited amount of pure protein. The remainder of this protein was used for amino acid sequencing. Lane 3 contains 2 μg of the crude protein extract of SRI101, an Ado kinase-deficient strain of *M. smegmatis* mc^2155. Lanes 4 and 5 each contain 2 μg of different preparations of SRICK1 with cloned Ado kinase, and lane 6 contains molecular mass markers.

Note: From "Identification and Characterization of a Unique Adenosine kinase from *Mycobacterium tuberculosis*" by M.C. long, V. Escuyer, and W.B. Parker, 2003, *Journal of Bacteriology*, 185 (22), p. 6548-55. Copyright 2003 by ASM Press. Reprinted with permission.

Amino acid sequence and gene identification

Protein sequencing of purified *M. tuberculosis* Ado kinase revealed that the 15 N-terminal amino acids were TIAVTGSIATDHLMR. A BLAST search (www.ncbi.nlm.nih.gov/BLAST) of the N-terminal sequence against the translated *M. tuberculosis* genome showed complete identity with the N-terminal sequence of a 324 amino acid protein with a molecular weight of 34,341 Da. The protein identified, Rv 2202c (Accession Q10391), is annotated as a hypothetical sugar kinase and is coded for by a gene currently named *cbhK*. From this point on, this gene will be referred to as *adoK*.

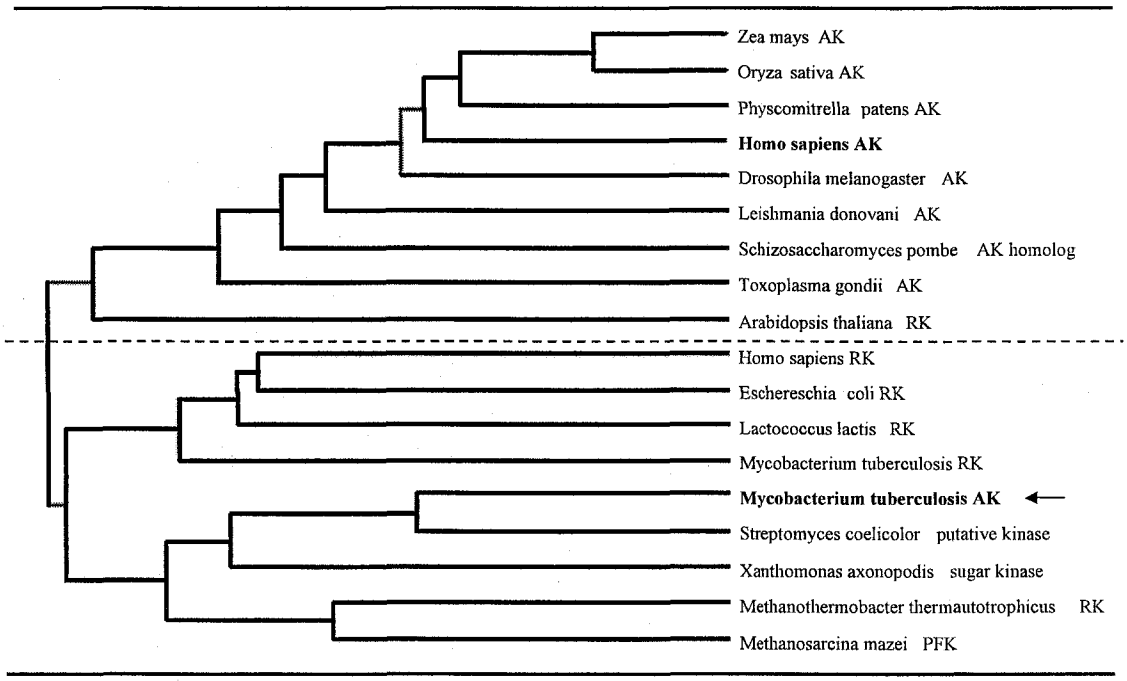
M. tuberculosis AK shared low (24%) overall homology with other known Ado kinases, which prevented the identification of the function of this enzyme based on its amino acid sequence. In light of its low sequence homology with other Ado kinases, a phylogenetic analysis of this enzyme was performed. The amino acid sequence from *M. tuberculosis* Ado kinase was aligned with sequences from related Ado kinases and ribokinases, and a phylogenetic tree was created based on this alignment. The phylogenetic tree indicated that *M. tuberculosis* Ado kinase was more closely related to ribokinases from various sources than other Ado kinases (Figure 5).

Amino acid sequence alignment permitted identification of some regions in the *M. tuberculosis* enzyme that are highly conserved with other Ado kinases (Figure 6). Analysis of the aligned sequences revealed that one region exists from D250 to L263 of *M. tuberculosis* Ado kinase that is homologous with the region that interacts with the γ-phosphate

of ATP in the human enzyme [79]. This region had 9/14 identical residues between human and *M. tuberculosis* Ado kinase, with 11/14 overall homology between sequences. Unlike the amino acids that interact with ATP, those that interact with Ado are mostly scattered in the N-terminal half of the sequence and do not form a highly conserved linear motif. Sequence alignment with human Ado kinase revealed several conserved amino acid residues that interact with Ado in the active site [79]. The amino acid sequence also contained an NXXE motif that is highly conserved in the PfkB family of carbohydrate kinases, and has been identified as being important for binding pentavalent ions and Mg^{2+} in the active site [85].

According to BLAST searches, the amino acid sequence of *M. tuberculosis* Ado kinase shared at least 50% sequence homology with proteins predicted from *Frankia spp.*, *Nocardia spp.*, *Streptomyces spp.*, *Burkholderia spp.*, *Desulovibrio vulgaris, Methylococcus capsulatus, Thiobacillus ferrooxidans, Bordetella spp.*, *Xanthomonas campestri,* and other *Mycobacterium spp.* These proteins either have unspecified functions or predicted functions as carbohydrate or sugar kinases.

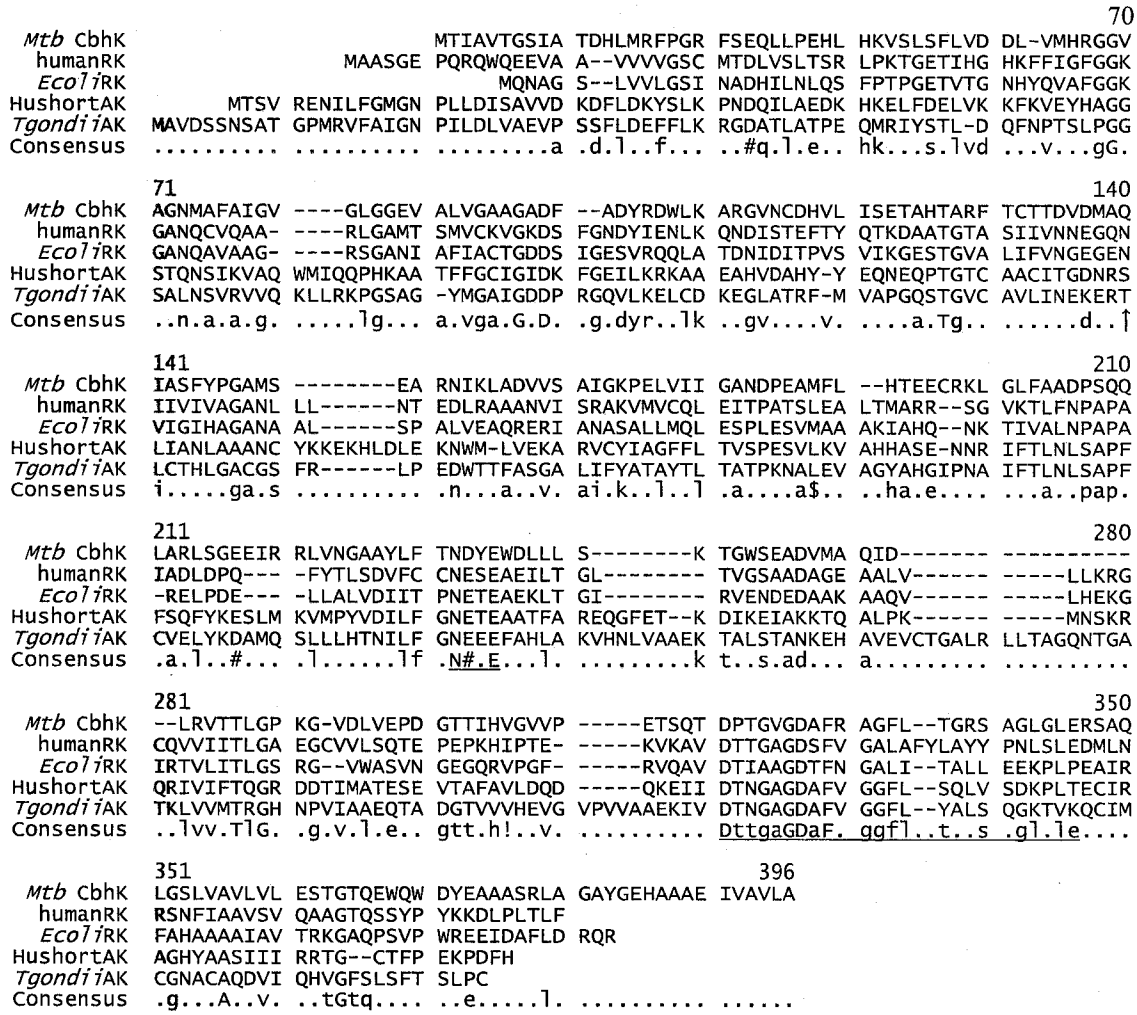
Figure 5.



Phylogenetic analysis of *M. tuberculosis* Ado kinase and related enzymes from the PfkB family. Amino acid sequences of several Ado kinases (AK) and ribokinases (RK) from various sources were aligned with phosphofructokinase (PFK) from *Methonosarcina mazei*, a *Streptomyces coelicolor* putative kinase (the most closely related enzyme according to BLAST genome searches), and sugar kinase from *Xanthomonas axonopodis*. All proteins were members of the PfkB family of carbohydrate and nucleoside kinases. The phylogenetic tree was created as described in the materials and methods section. The dashed line roughly separates Ado kinases from ribokinases.

Note: From "Identification and Characterization of a Unique Adenosine kinase from *Mycobacterium tuberculosis*" by M.C. long, V. Escuyer, and W.B. Parker, 2003, *Journal of Bacteriology*, 185 (22), p. 6548-55. Copyright 2003 by ASM Press. Reprinted with permission.

Figure 6.



Amino acid sequence alignment of Ado kinase from *M. tuberculosis* (*Mtb* CbhK) with human ribokinase and Ado kinase, *E. coli* ribokinase, and *T. gondii* Ado kinase. This sequence alignment was created using Multalin software (http://www.toulouse.inra.fr/multalin/multalin.html) as described in the materials and methods section. The consensus sequence is shown below the aligned sequences. Lower case letters in the sequence indicate sites of amino acid homology, whereas uppercase letters indicate identically conserved amino acids; # indicates an acidic amino acid, Asp (D) or Glu (E); ! indicates an Ile (I) or Val (V), both hydrophobic amino acids; ↑ indicates the Gln (Q) which was mutated to Glu (E) in SRICK1; and the underlined amino acid stretch has been identified as the P-loop motif which interacts with the γ-phosphate group of ATP.

Characterization of cloned Ado kinase

To confirm that *Rv2202c* encoded an Ado kinase, the gene was cloned and expressed in Ado kinase-deficient strains of *M. smegmatis* and *M. tuberculosis*, SRI101 and SRICK1, respectively. SRI101 was rendered ado kinase-deficient by transposon mutagenesis [52]. Following transformation with pVV16/*Rv2202c*, Ado kinase activity was restored in both deficient bacterial strains to a level at least 900 times higher than the original mutants (Table 3).

While *M. tuberculosis* strain H37Ra is sensitive to methyl-Ado, Ado kinase-deficient mutants are methyl-Ado resistant, presumably because it lacks Ado kinase activity. Transformation of SRICK1 with pvv16/*Rv2202c* plasmid DNA restored its sensitivity to methyl-Ado (Table 3). Sensitivity of each *M. tuberculosis* strain to methyl-Ado corresponded with the amount of Ado kinase activity detectable in protein extracts from each *M. tuberculosis* strain (Table 3). Denaturing SDS-PAGE of crude protein extracts from complemented bacterial strains revealed a prominent protein band at 35 kDa (Figure 4), while size-exclusion chromatography revealed that cloned *Rv2202c* eluted at 136 kDa, the same as native Ado kinase (data not shown). Mass spectrometry of cloned Ado kinase demonstrated that the protein was 34,337 Da, confirming the fidelity of the construct (data not shown). Cloned Ado kinase purified in exactly the same manner as the native enzyme, and kinetic studies with cloned Ado kinase demonstrated that the K_m values for Ado and methyl-Ado were the same as those of the native enzyme (Table 4). Restoration of Ado kinase activity and methyl-Ado sensitivity to SRICK1 and SRI101 cells that had

been complemented with *Rv2202c* confirmed that *Rv2202c* was the gene that codes for Ado kinase in *M. tuberculosis*.

The second Ado kinase clone that was created had a polyhistidine tag. The fidelity of the cloned gene was confirmed by DNA sequencing. Protein was expressed as described above and the expression yield was about 20 mg/L of cell culture. On native gels, the protein migrated as a single band, indicating that it was uniformly folded in solution. Expressed protein had a specific activity that was similar to that of purified native Ado kinase, indicating that the affinity tag did not negatively effect Ado kinase activity.

Characterization of the SRICK1 adoK gene

SRICK1 is an Ado kinase-deficient spontaneous mutant that was selected by incubating *M. tuberculosis* H37Ra on LB agar containing 10 μM methyl-Ado [32]. In order to identify mutations to the *adoK* gene that may render the protein inactive, the *adoK* gene was amplified by PCR and the DNA was sequenced. One C→G mutation at position 334 in the *adoK* gene was identified, which translates to a Gln112Glu mutation in the protein (Figure 6). It is possible that this point mutation is responsible for the loss of Ado kinase activity.

Table 3.

Ado kinase activity and MIC values for methyl-Ado in different strains of *M. tuberculosis* and *M. smegmatis*

Strain	Properties	Ado kinase activity[a] (nmol/mg-min)	MIC for methyl-Ado (μg/ml)
M. tuberculosis strains:			
H37Ra	wild-type	5	5
SRICK1	Ado kinase-deficient	<0.2	>50
SRICK1-pVV16/*adoK*	SRICK1 transformed with *adoK*	100	1
M. smegmatis strains:			
mc^2155	wild-type	15	ND
SRI101	Ado kinase-deficient	0.4	ND
SRI101-pVV16/*adoK*	SRI101 transformed with *adoK*	360	ND

a) Ado kinase activity was measured in crude protein extracts of the indicated bacterial strain.

ND, not determined

Table 4.

Comparative properties of *M. tuberculosis* and human Ado kinases

Property	*M. tuberculosis* Ado kinase	Human Ado kinase (reference)
Molecular weight (kDa)	34.3	38.7 [63, 82]
Stoke's radius (Å)	49.4	26.4 [82]
Quaternary Structure	dimer	Monomer [66, 82]
pH optimum	8-11	5.5, 7.5-8.5 [63]
pI	4.5	5.9 [82]
K_i iodotubercidin (nM)	210 ± 100	3-30 [71, 91]
K_m Ado (μM)	0.80 ± 0.08	0.15-0.4 [66, 67, 92]
K_m ATP (μM)	1400 ± 460	75 [66, 92]
K_m mAdo (μM)	79 ± 26	961 ± 385
V_{max} mAdo (nmol/mg-min)	72 ± 3.9	0.64 ± 0.21
Catalytic efficiency - mAdo	0.91	0.00067
Phosphate donors	dGTP>GTP>ATP>dATP, UTP, TTP >>CTP, dCTP	GTP, dGTP> ATP, dATP [29] >CTP, UTP >TTP, dCTP
Substrates	Adenosine	Adenosine>>deoxyadenosine [29]

a) References to properties of human adenosine kinase, numbers indicate bibliographic references. Properties of *M. tuberculosis* AK were generated as a result of this work.

Note: From "Identification and Characterization of a Unique Adenosine kinase from *Mycobacterium tuberculosis*" by M.C. long, V. Escuyer, and W.B. Parker, 2003, *Journal of Bacteriology*, 185 (22), p. 6548-55. Copyright 2003 by ASM Press. Reprinted with permission.

Characterization of native Ado kinase.

Substrate specificity. Kinase activity was evaluated with 20 μM Ado, guanosine, inosine, deoxyadenosine, cytidine, uridine, or thymidine. Of the nucleosides tested, Ado was the only nucleoside that served as a substrate for this kinase (Table 5). In the presence of other nucleosides, kinase activity was below the detection limit (3% of the activity seen with Ado, data not shown), which indicated that Ado was at least 30-fold better than other nucleosides as a substrate for this enzyme. Because deoxyadenosine is a substrate for human Ado kinase with 7% of the activity of Ado at 25 μM [66], deoxyadenosine was further examined as a substrate for *M. tuberculosis* Ado kinase. Assay conditions were altered by increasing protein concentration so that the detection limit was 500-fold higher than for Ado; under these conditions, deoxyadenosine exhibited 0.014 ± 0.004% of the activity of Ado (data not shown), which indicated that it was at least 7000-fold worse than Ado as a substrate.

Table 5.

Adenosine kinase activity[a] in the presence of natural nucleosides

	M. tuberculosis		human[b][66]
Substrate	**20 μM**	**25 μM**	**25 μM**
Adenosine	100	100	100
Guanosine	<3	ND	ND
Inosine	<3	ND	1
Cytidine	<3	ND	ND
Uridine	<3	ND	ND
Thymidine	<3	ND	ND
Deoxyadenosine	<3	0.014	2

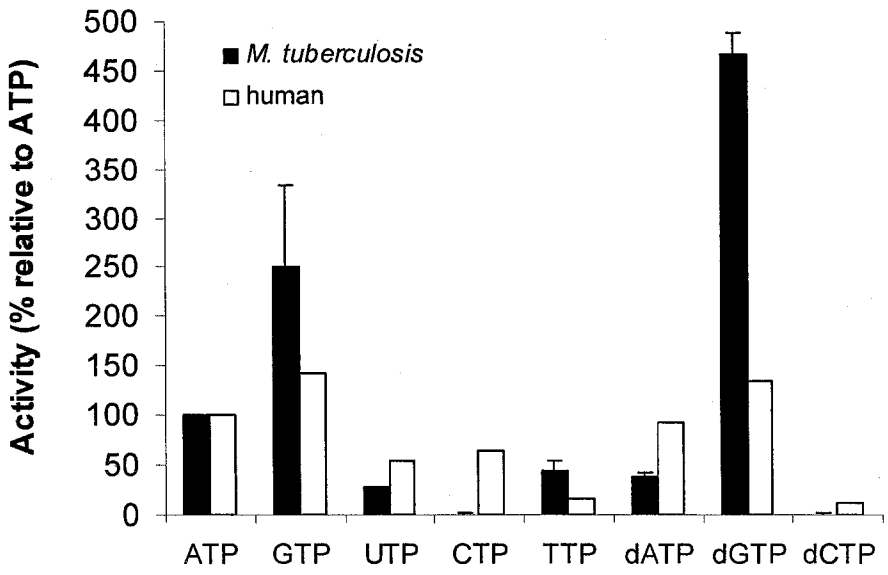
a. Activity is expressed as a percentage relative to adenosine
b. Results are from the indicated reference
ND, not determined

In order to eliminate the possibility that the Ado kinase activity that we detected was an artifact of a related sugar kinase, ribose, glucose, fructose, and fructose-6-phosphate were evaluated as inhibitors in the presence of 20 μM Ado. None of the sugars assayed were inhibitors at 1000 μM (data not shown), which indicated that these compounds were at best poor substrates and suggested that this protein is a specific Ado kinase.

Phosphate donors. Potential phosphate donors, including ATP, GTP, UTP, CTP, TTP, dATP, dGTP and dCTP, were tested at 5 mM each. GTP and dGTP were the best phosphate donors with 2.5 and 4.7-times the activity observed with ATP, respectively (data not shown). UTP, dATP, and TTP exhibited about 33% of the activity observed with ATP, while CTP and dCTP were the worst phosphate donors with less than 0.5% and 0.2% of the activity observed with ATP respectively (Figure 7).

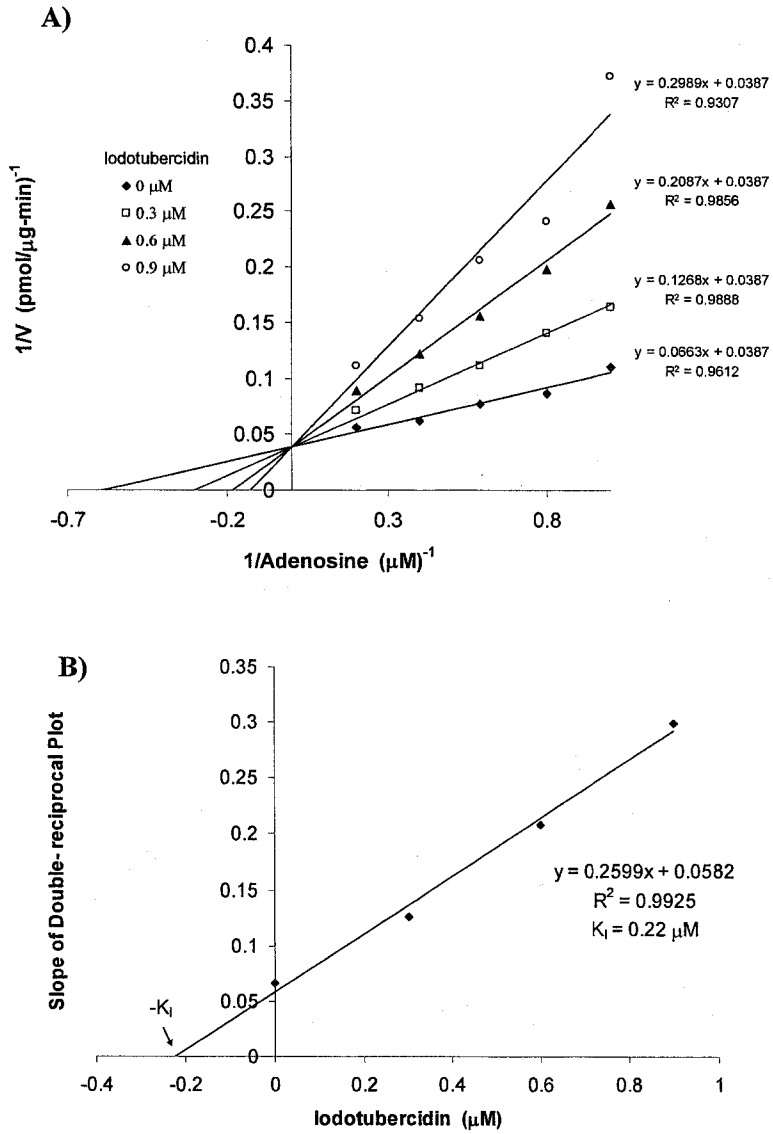
Iodotubercidin as an inhibitor of Ado kinase. Iodotubercidin is an Ado analog that is a potent competitive inhibitor of human Ado kinase with a K_i between 3 and 30 nM [71, 93]. Double-reciprocal plots of Ado kinase activity in the presence of iodotubercidin were constructed with five data points for each concentration of iodotubercidin (regression coefficients were at least 0.94, Figure 8a). These showed that iodotubercidin was a competitive inhibitor of *M. tuberculosis* Ado kinase. The K_i was determined to be 210 ± 100 nM (n=3) from replots of 1/slope of the double-reciprocal plot *vs* the concentration of iodotubercidin (regression coefficients were at least 0.99, Figure 8b).

Figure 7.



Evaluation of potential phosphate donors for *M. tuberculosis* and human Ado kinases. Ado kinase activity was assayed in the presence of 5 mM phosphate donors. These assays included 10 mM KCl. Kinase activity is portrayed relative to the activity measured in the presence of ATP as a phosphate donor. Values for human Ado kinase are from literature [66].

Figure 8.

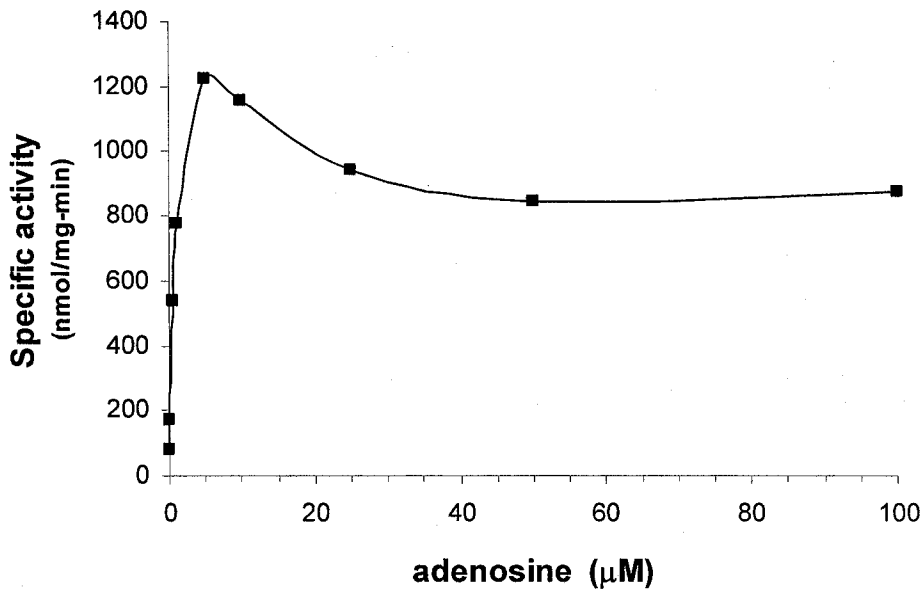


Evaluation of iodotubercidin as an inhibitor of Ado kinase from *M. tuberculosis*. Ado kinase was assayed in the presence of 0 to 900 nM iodotubercidin. A) Lineweaver-Burke double-reciprocal plots were created and B) the K_i was extrapolated from a plot of the slope of the double-reciprocal plot vs. concentration of iodotubercidin. Experiments were performed three times. The graphs above are from a single experiment.

Substrate inhibition. Substrate inhibition is a common characteristic of Ado kinases from various sources. *M. tuberculosis* Ado kinase reaction velocity was evaluated in the presence of 0.05 to 100 μM Ado. Reaction velocity increased linearly up to 0.5 μM and reached a maximum at 5 μM Ado, then decreased with increasing concentrations of Ado. By 25 μM, the reaction rate had decreased to 75% of the maximum rate, and reaction velocity plateaued at 70% of the maximum velocity from 50 to 100 μM Ado (Figure 9). This substrate inhibition is similar to that reported for human Ado kinase [92].

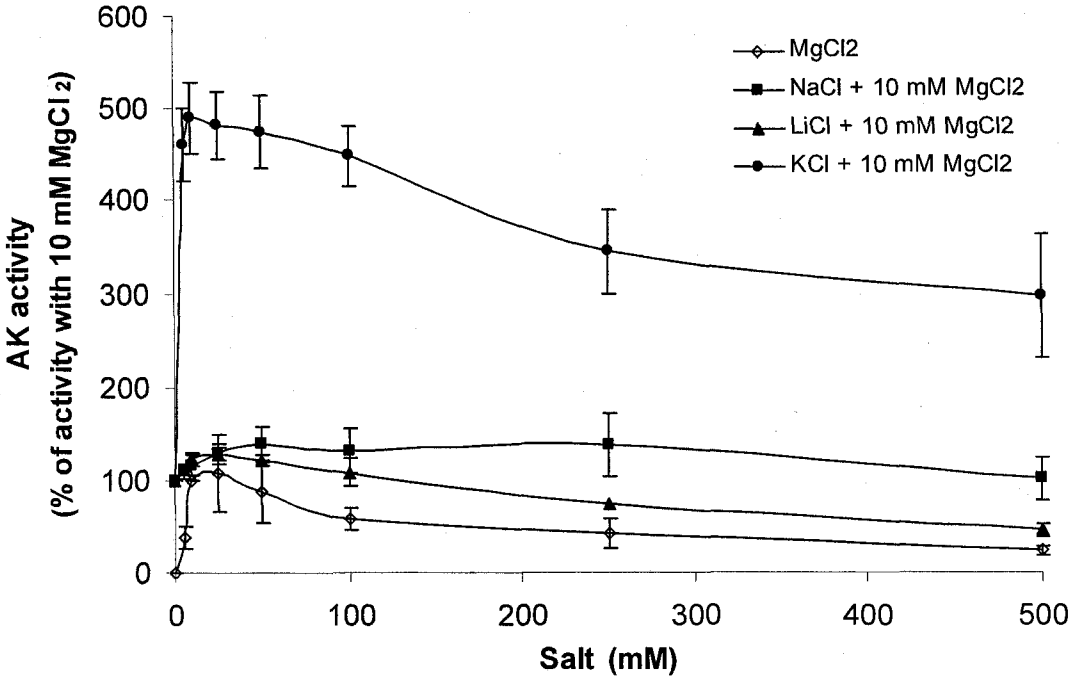
Ion dependency. *M. tuberculosis* Ado kinase was dependent on the presence of Mg^{2+} for activity. Optimum Ado kinase activity occurred in the presence of 10 to 50 mM $MgCl_2$; however, $MgCl_2$ concentrations greater than 50 mM were inhibitory (Figure 10). A 50% loss of Ado kinase activity occurred with 125 mM $MgCl_2$, corresponding to an ionic strength of 375 mM. A similar decrease in activity did not occur until 750 mM NaCl in the presence of 10 mM $MgCl_2$, corresponding to an ionic strength of 780 mM. Therefore, the loss of activity appears to be a specific effect of Mg^{2+}, and not an effect of ionic strength. The ATP:Mg ratio plays a critical role in regulating activity for Ado kinases from various sources [60, 61, 66, 92] and *M. tuberculosis* Ado kinase assays were typically performed with an ATP:Mg ratio of 1:2.

Figure 9.



Ado kinase activity in the presence of increasing Ado concentrations. Ado kinase activity was assayed as described in the materials and methods section.

Figure 10.



Effect of salts on Ado kinase activity. Ado kinase activity was assayed in the presence of increasing concentrations of various salts. Activity was dependent on $MgCl_2$, therefore assays with monovalent cations (Na^+, Li^+, and K^+) included 10 mM $MgCl_2$.

M. tuberculosis Ado kinase activity was assessed in the presence of the monovalent salts LiCl, NaCl, and KCl, and the enzyme exhibited stimulation in the presence of KCl that has not been reported for other Ado kinases. In the presence of 10 mM KCl, the K_m *(app)* for Ado decreased from 3.4 μM to 0.8 μM ($p<0.05$), and the V_{max} increased from 60 to 180 nmol/mg-min ($p<0.005$). A profound effect was also observed for methyl-Ado in the presence of 10 mM KCl. The K_m*(app)* decreased from 709 to 79 μM ($p<0.005$), while the V_{max} increased from 2.3 to 72 nmol/mg-min ($p<0.005$). The effect of KCl on the phosphorylation of Ado and methyl-Ado resulted in 12-fold and 300-fold increase in V_{max}/K_m, respectively, which indicated that Ado kinase phosphorylated these substrates more efficiently in the presence of KCl. The stimulatory effects were observed in the presence of KCl, but not in the presence of NaCl or LiCl (Figure 10). Therefore, we attributed this stimulation of activity to a specific effect of K^+.

Methyl-Ado was evaluated as a substrate for human Ado kinase in the presence of 40 mM KCl, a concentration that was representative of most human Ado kinase assays. With human Ado kinase, the K_m *(app)* for methyl-Ado was 960 μM, and the V_{max} was 0.64 nmol/mg-min (Table 4), which are similar to the values seen for *M. tuberculosis* Ado kinase in the absence of KCl.

Inorganic phosphate. P_i has been reported to have a stimulatory effect on various Ado kinases, with a concurrent depression of the K_m for Ado and ATP, and increase in V_{max} for these reactants [83-85, 94]. The effect of P_i on *M. tuberculosis* Ado kinase was evalu-

ated using up to 50 mM sodium phosphate, with Ado concentrations both above and below the K_m for Ado, and little or no stimulation of activity was observed (Figure 11).

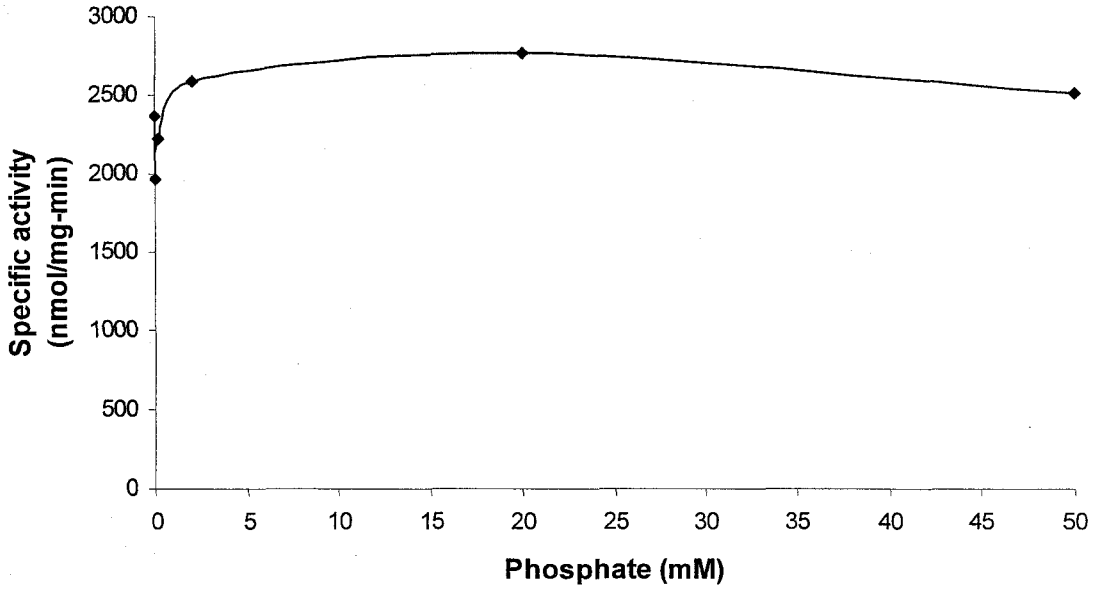
Determination of optimum pH. *M. tuberculosis* Ado kinase activity was dependent on pH. The optimum pH range for Ado kinase activity was from pH 8 to 11 (Figure 12), with activity decreasing rapidly at pH values above pH 11 and below pH 8. Ado kinase was inactive at its calculated isoelectric pH 4.3, and activity was not restored by dialyzing enzyme back to pH 8. Although the enzyme is stable over time, it cannot tolerate dramatic reductions in pH.

Determination of the quaternary structure of Ado kinase. The quaternary structure of the protein was difficult to determine since different methods yielded different results. Ado kinase eluted from a Superose 12 size exclusion column as a single peak near the aldolase standard (158 kDa), and absorbance at 280 nm correlated with Ado kinase activity. The molecular weight of native Ado kinase was determined to be 136 kDa, with a Stoke's radius of 49.4Å from the calibration curve (R = 0.99, Figure 3). However, native gels revealed that the enzyme ran near its monomer molecular mass [88]. Since the results of size exclusion chromatography were very different from native gels, light scattering and analytical ultracentrifugation were used for further native molecular mass determinations.

Light scattering analysis of the peak of Ado kinase activity as it eluted from a Superose 12 column indicated that the molecular weight of the protein was 74,840 ± 710

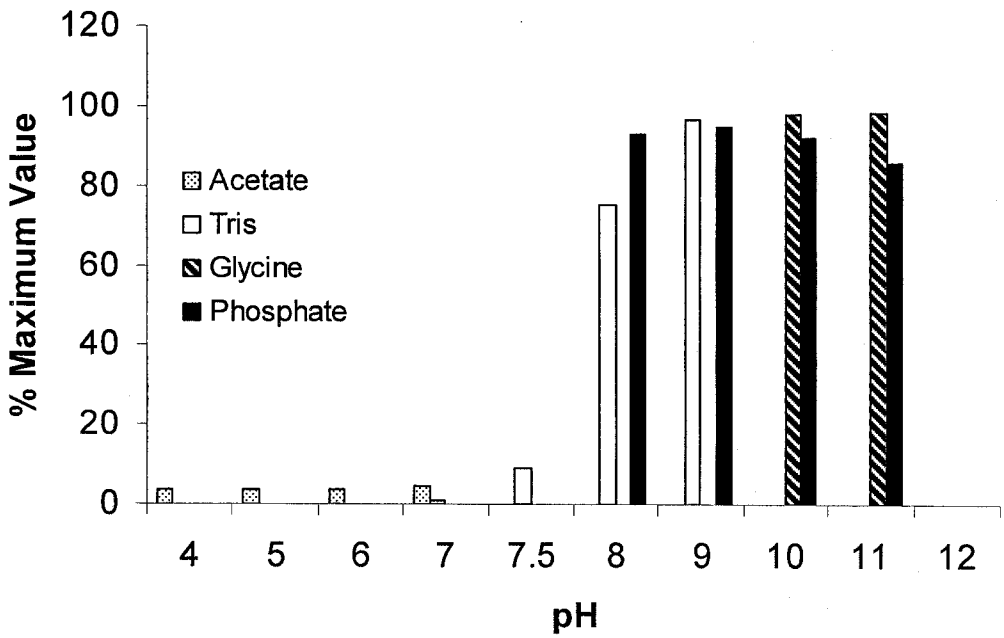
kDa (N = 4, Figure 13), this molecular weight is approximately the molecular weight of a dimer. The molecular weight distribution across the width of the peak was uniform, as indicated by the line traversing the peak of refractive index (RI), 15° light scatter, and 90° light scatter (Figure 13). Sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation confirmed that the native form of the enzyme was a dimer with a molecular weight of around 70 kDa, and a sedimentation coefficient of 4.47. The dissociation constant for the dimer was 2.3×10^{-7} M, and weight-fraction plots demonstrated that >90% of the enzyme was a dimer at 1 mg/ml (~30 μM; data not shown).

Figure 11.



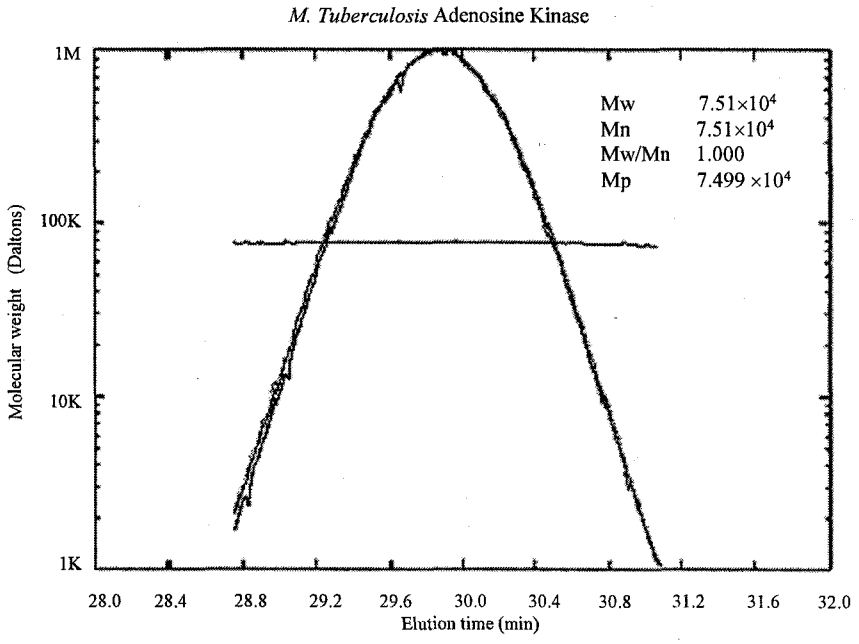
Effect of inorganic phosphate on Ado kinase activity. Ado kinase activity was evaluated in the presence of 0 to 50 mM sodium phosphate. The graph represents an average of two experiments.

Figure 12.



Determination of the optimum pH for Ado kinase activity. Ado kinase activity was determined in the presence of 50 mM buffers spanning the pH range from 4 to 12. Values are presented as a percentage of the maximum activity measured.

Figure 13.



Native molecular mass of Ado kinase as determined by light scattering. Refractive indexes, 15° and 90° light scattering data were acquired for Ado kinase as it eluted from a Superdex 200 size exclusion column. The scale on the ordinate axis is a reference for the molecular mass distribution line; the refractive index, 15° light scatter and 90° light scattering traces are scaled to the maximum height of the plot. The plot of elution time versus molecular mass shows the distribution of molecular mass across the Ado kinase peak. M_w is the weighted-average molecular mass, Mn is the average molecular mass, and Mp is the molecular mass associated with the maximum peak height.

Note: From "Identification and Characterization of a Unique Adenosine kinase from *Mycobacterium tuberculosis*" by M.C. long, V. Escuyer, and W.B. Parker, 2003, *Journal of Bacteriology*, 185 (22), p. 6548-55. Copyright 2003 by ASM Press. Reprinted with permission.

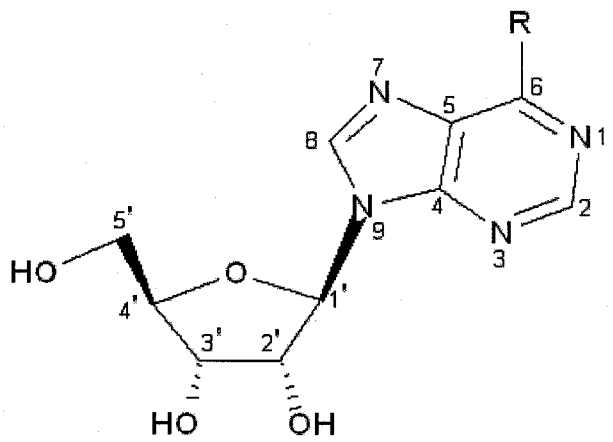
Structure-activity relationship for *M. tuberculosis* Ado kinase.

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, 149 modified Ado analogs were evaluated containing either endocyclic substitutions, exocyclic substitutions, or a combination of both on the adenine base or ribose moieties (Table 6). Figure 14 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 [95]. The purity of each compound was determined by reverse-phase chromatography. Most compounds were >90% pure. If a compound was <70% pure, it was rejected from this SAR. 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 (Figure 15), 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 [59, 86, 96, 97]. 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. The following results summarize our data found in table 6.

Figure 14.

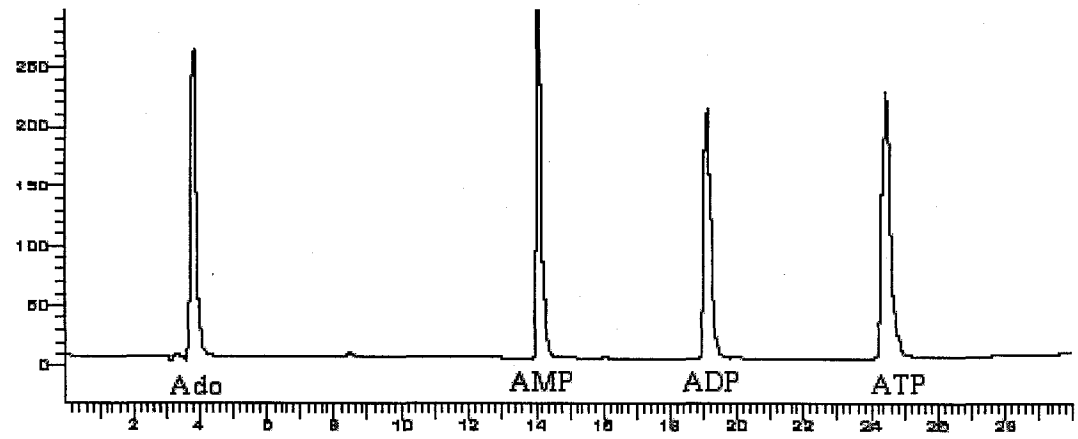


R	Name
H-	Purine riboside
NH_2-	Adenosine

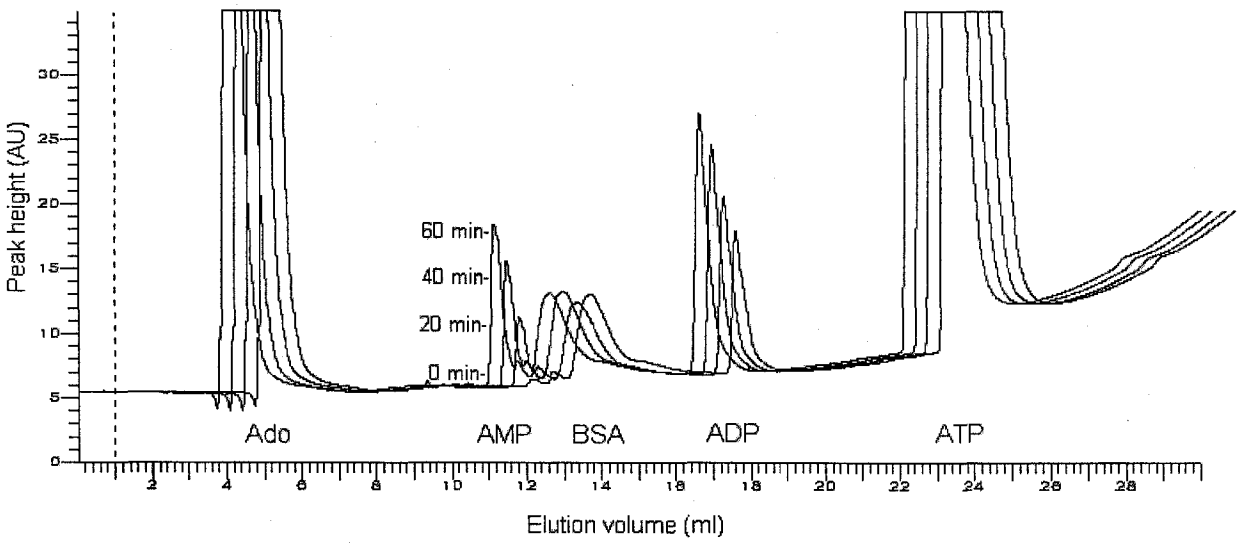
Structure and numbering convention for Ado and purine riboside.

Figure 15.

A)



B)



BioBasic anion exchange HPLC of Ado and its phosphorylated products. A) Chromatographic separation of 20 μM each of Ado, AMP, ADP, and ATP standards. B) Ado kinase was assayed as described in the materials and methods section. The overlay of this typical timecourse demonstrates the phosphorylation of Ado by *M. tuberculosis* Ado kinase at 1, 20, 40, and 60 min. Timepoints are offset by a 3˚ rotation.

Note: From "Structure-activity relationship for nucleoside analogs as inhibitors or substrates of adenosine kinase from Mycobacterium tuberculosis" by M.C. Long and W.B.

Modification of the purine base

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

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 substrates were Ado-N^1-oxide **(2)** and N^1-methyl-Ado **(3)** 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 [97].

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. Size may dictate the function of compounds

with N^1-substitutions. For example, N^1-methyl-Ado was a substrate but a poor inhibitor, whereas N^1-ethyl-Ado was a potent inhibitor but not a substrate. The trend was for compounds with large exocyclic substitutions at N^1 to be good inhibitors, but bad substrates. Indeed, N^1-benzyl-Ado **(5)** was a potent competitive inhibitor with a K_i of 0.19 ± 0.1 μM, but not a substrate.

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 2-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 [59, 97]. 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 [59, 97-100].

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.

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.

Of the six halogen-substituted analogs of deaza-Ado that have been analyzed, the best substrates for *M. tuberculosis* Ado kinase were 2,3-difluoro-3-deaza-Ado **(24)**, 2-fluoro-3-deaza-Ado **(22)**, and 3-fluoro-3-deaza-Ado **(23)**, with specific activities of 81, 63, and 16 nmol/mg-min, respectively. For human Ado kinase, the best substrates were 2,3-fluoro-3-deaza-Ado **(24)**, 2,3-difluoro-3-deaza-7-iso-Ado **(25)**, and 3-fluoro-3-deaza-Ado **(23)**, with specific activities of 6, 5, and 1 nmol/mg-min, respectively. With the exception of 2,3-difluoro-3-deaza-7-iso-Ado **(25)**, compounds that were substrates for *M. tuberculosis* Ado kinase were less active with human Ado kinase. This finding is consistent with the lack of activity of deaza-Ado with mammalian Ado kinase [59, 101-103].

The agent that demonstrated the most inhibition of *M. tuberculosis* Ado kinase was 3-chloro-3-deaza-Ado **(27)**. The difference in substrate activity between 3-fluoro-3-deaza-Ado **(23)** and 3-chloro-3-deaza-Ado **(27)** (16 and <0.2 nmol/mg-min, respectively) implied that steric hindrance may exclude exocyclic modifications as large as a chloro group at the 3-position of Ado. However, measurement of the inhibitory effects of these agents seemingly contradict this hypothesis, since it is likely that 3-chloro-3-deaza-Ado **(27)** binds to the active site of the enzyme in order to inhibit activity. Since the active site has Ado and ATP binding domains, it is possible that 3-chloro-3-deaza-Ado **(27)** interacts with the ATP-binding domain, thereby acting as a competitive inhibitor of ATP, or that 3-chloro-3-deaza-Ado **(27)** binds more tightly to the Ado-binding domain than 3-fluoro-3-deaza-Ado **(23)** and that this interaction makes it a better inhibitor than a substrate of this enzyme. These results indicate that a nucleophilic substitution is preferred at the 3-position of deaza-Ado, possibly to act as a hydrogen bond acceptor for substrate

recognition, and that increasing the size of the nucleophilic substitution beyond the size of a fluorine atom increases the chance that the compound will be an inhibitor rather than a substrate.

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 **(29-31, 35, and 36)** were excellent substrates with as much or more activity than Ado with the exception of 6-methyl-purine riboside **(35)** 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 [59, 97]. 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 [59, 97]. This disparity may reflect differences in conditions for assaying these compounds.

In *M. tuberculosis* Ado kinase, N^6-amino-Ado **(28)** demonstrated a >99% decrease in activity. Purine riboside **(29)** and its 6-substituted analogs **(30-41)** 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 **(30)** and 6-bromo-purine riboside **(31)**. However, compounds of similar size and electronegativity such as 6-oxy-purine riboside **(33)** or 6-mercapto-purine riboside **(34)**

were not substrates since the modification resembled the lactam (keto) tautomeric form. These results were similar to observations made with *T. gondii* Ado kinase [86]. Of the 6-position substitutions made to purine riboside (**29**, 66 nmol/mg-min), 6-chloro, -bromo, and -methyl **(30, 31 and 35)** groups were at least as active as purine riboside **(29)** 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 **(37)**. N^6-amino-adenosine **(28)** was similar in size to 6-methoxy-purine riboside **(37)** 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 Ado kinase. These inhibitors fell into one of two categories. The first category included compounds in which the substitution was at least 3-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 **(39)**, demonstrated a mixed mode of inhibition and a K_i of 0.15 ± 0.08 μM.

7-position. 7-deaza-Ado **(52)** and its analogs are excellent substrates for human, rabbit, and *T. gondii* Ado kinases and are known as excellent inhibitors as well [38, 48, 59, 71, 93, 104]. This series of compounds also proved to be excellent inhibitors of *M. tuberculosis* Ado kinase, with 7- iodo-7-deaza-Ado **(53)** and 7-cyano-7-deaza-Ado **(54)** 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.

8-position. Consistent with previous reports, 8-aza-Ado **(56)**, was a good substrate for human Ado kinase, maintaining 38% of the activity of Ado [59, 97, 98]. 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 **(60)**, was as active as 8-aza-Ado **(56)** and improved the selectivity for *M. tuberculosis* Ado kinase relative to 8-aza-Ado **(56)**. Likewise, exocyclic substitutions to the 8-position proved to be poor inhibitors, whereas the addition of an endocyclic *N*, 8-aza-ado **(56)** and 2-fluoro-8-aza-Ado **(60)**, proved to be good inhibitors.

9-position. The endocyclic substitution at the 9-position, 9-deaza-Ado **(74)**, 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 **(74)** proved to be a good inhibitor.

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, **71**) proved to be one of the

best substrates for *M. tuberculosis* Ado kinase of all of the compounds tested, with a specific activity of 3800 nmol/mg-min (95 % of the activity of Ado). Although they were not as active as Formycin A **(71)**, 2-fluoro-formycin A **(72)** and 2-amino-formycin A **(73)** were also excellent substrates for the *M. tuberculosis* enzyme. Formycin A **(71)** 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 [59]. Formycin A **(71)** 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.

Alternative base structures

Of the thirteen ribosides tested that had a pyrimidine-base or alternative ring structures **(137-149)**, most were not substrates for Ado kinase with specific activities <2 nmol/mg-min. One exception to this was ribavirin **(140)**, 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 [74]. 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 **(147)**.

Glycosidic bond position

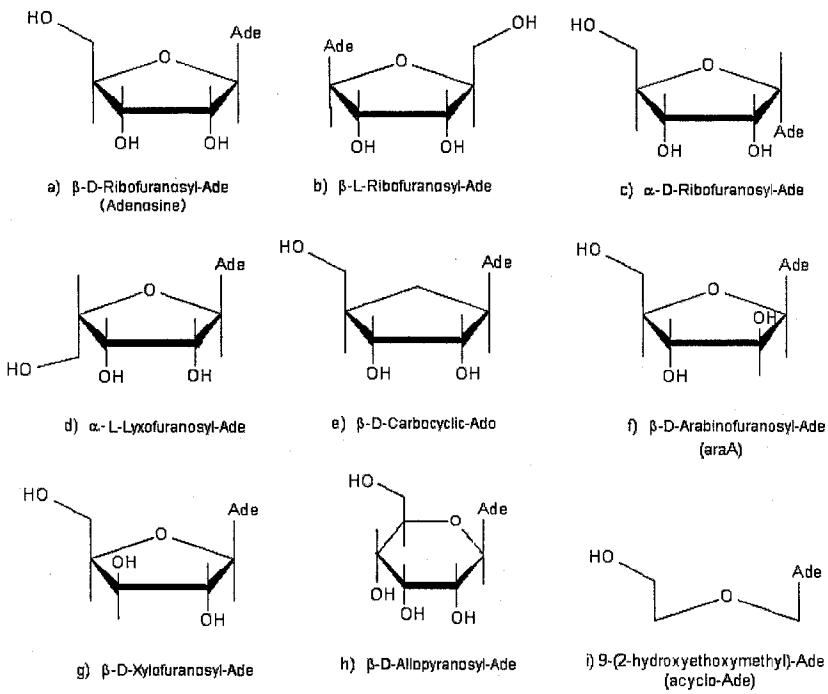
Most of the adenine ribonucleoside and purine ribonucleoside analogs tested had glycosidic bonds at the 9-position of the purine base. Three compounds were tested that had the glycosidic bonds at an alternative position. Of these, two had additional modifica-

tions. These compounds are considered to have major structural modifications relative to Ado. 3-[β-D-ribofuranosyl]-adenine **(78)** was slightly active in *M. tuberculosis* Ado kinase (~1% of the activity of Ado), whereas it maintained 11% of the activity of Ado in human Ado kinase, consistent with a previous report [97]. Compounds which had other modifications in addition to he glycosidic bond, 7-[α-D-ribofuranosyl]-adenine **(79)** and 8-aza-8-[β-D-ribofuranosyl]-adenine **(80)**, were both inactive in this system with respective activities of <2 and <1 nmol/mg-min.

Modification to the ribofuranosyl moiety

Fifty-six compounds were tested that had single or multiple changes to the sugar moiety of Ado, or combinations of changes to the sugar and base. *M. tuberculosis* AK phosphorylated only sugars in the furan conformation and excluded all twelve pyranoses tested **(125-136)**. Furthermore, 9-(2-hydroxyethoxymethyl)-adenine (acyclo-adenosine, **123**) and 9-[2-[(2-hydroxyethyl)-methylamino]-ethoxymethyl]-adenine **(124)** were not substrates or inhibitors of *M. tuberculosis* Ado kinase. Several of the sugar structures are shown in Figure 16. Following is a summary of the results of changes to the ribofuranosyl moiety.

Figure 16.



Structures of some of the sugar moieties utilized in the structure-activity relationship.

2'-position. Consistent with previous observations with human Ado kinase, 2'-deoxy-Ado **(87)** was a poor substrate [91, 97]. The enzyme had a preference for the 2'-OH to be trans to the adenine moiety as illustrated by the <99% decrease in activity seen with 9-[β-D-arabinofuranosyl]-adenine (araA, **93,** Fig 15f). When multiple substitutions were made, including combinations to the base and ribose moieties such as 2'-deoxy- 2,2'-difluoro-Ado **(91)** and 2-fluoro-araA **(94)**, no activity was detected.

M. tuberculosis Ado kinase also preferred the 2'-hydroxyl group to be trans to the adenine moiety, as demonstrated by the <99%-loss in activity measures with araA **(93)**. When substitutions to the base are combined with substitutions at the 2-position as in the case of 2-fluoro-araA **(94)** and 2'-deoxy-2,2'-difluoro-Ado **(91)**, 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 **(88)** 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 **(90)** was the only compound with a 2'-substitution that demonstrated noteworthy inhibition with 87% of the activity of Ado at 10 μM. All others were either poor inhibitors or did not inhibit *M. tuberculosis* Ado kinase at all.

3'-position. For *M. tuberculosis* Ado kinase, the presence of a 3'-OH group trans to the adenine moiety was necessary for substrate activity. Modification of the 3'-hydroxyl group had a greater impact on substrate activity than modification of the 2'-hydroxyl

group. 3'-deoxy-Ado **(98)** had activity of <1 nmol/mg-min, whereas changing the 3'-hydroxyl group to the cis-conformation, as in the case of 9-[β-D-xylofuranosyl]-Ade (Fig 15g, **101**), resulted in a specific activity of 1 nmol/mg-min. Furthermore, no compounds with combinations of 2' and 3' substitutions 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. No compounds with substitutions 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.

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 15e, **82**) maintained about the same activity in both human and *M. tuberculosis* Ado kinases (37% and 38% of the activity of Ado, respectively). Although 2- amino-Ado **(13)** was similarly active in both Ado kinases, 2-amino-carbocyclic-Ado **(83)** was at least a 20-fold better substrate for *M. tuberculosis* Ado kinase than human Ado kinase. Activities measured with human Ado kinase were consistent with previous reports [105-107].

All of the carbocyclic compounds were at best poor inhibitors, inhibiting Ado phosphorylation by no more than 81% at 100 μM.

5'-position. There was some flexibility about the 5'-position, although these compounds maintained at most 4% of the activity seen with Ado. The cis-conformation of the 5'-C was not a requirement for either Ado kinase as demonstrated by 9-[α-L-lyxofuranosyl]-adenine (Fig 15d, **110**) with 4% and 15 % of the activity of Ado in *M. tuberculosis* and human Ado kinases, respectively. This compound differs from Ado only in that the 5'-C is trans to the adenine moiety. Other compounds with modifications to the 5'-position 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 **(112)** was about twice as active as 9-[β-D-5-methyl-(*talo*)-ribofuranosyl]-2-fluoro-adenine **(113)**. 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.

There was strong evidence that 5'-amino-5'-deoxy-Ado **(109)** was a substrate for *M. tuberculosis* Ado kinase, while it is known not to be a substrate for the mammalian homolog [59, 71, 93]. 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 (Figure 17). The ADP peak also increased with time (Figure 17). Since ADP was also a product of the reaction, the increase of this product provided indirect evidence that 5'-amino-5'-deoxy-Ado **(109)** 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, 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. 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 5'-position provided the most potent inhibitors of all of the sugar substitutions. Of these, 5'-amino-5'-deoxy-Ado **(109)** was a potent competitive inhibitor with a K_i of 0.8 ± 0.4 μM. This compound was more potent at inhibiting Ado phosphorylation than 5'-deoxy-Ado **(107)**. Furthermore, compounds that had the addition of a 5'-methyl group in the *talo*-conformation **(113 and 115)** were about 10-times less potent as inhibitors than their *allo*-conformers **(112 and 114)**.

The activity of 5'-amino-5'-deoxy-Ado **(109)** is noteworthy because this compound is known not to be a substrate for human Ado kinase; indeed, it is a potent inhibitor of this enzyme [59, 71, 86, 93]. 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 **(109)** and its phosphorylated product are warranted in light of these unusual results.

MIC assays

A group of nucleosides were selected for MIC analysis with *M. tuberculosis* H37Ra, SRICK1, and SRICK1::*adoK* (Table 7). Compounds were selected based on their unique structures or substrate activity, which ranged from <0.1 to 4030 nmol/mg-min. Several of these compounds were promising in terms of antimycobacterial activity, with MIC values similar to that of the ethambutol control. The specific activity of the compound as measured by our assays 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.

One reason why substrate activity may not be predictive for antitubercular activity is that Ado analogs are prone to deamination by Ado deaminase or cleavage by Ado hydrolase [39]. Indeed, for some compounds that did not demonstrate antitubercular activity, compounds were re-assayed with the inclusion of 5 μM dCF. When this Ado deaminase inhibitor was included, most of the compounds exhibited antitubercular activity (Ta-

ble 7). Normally an exocyclic substitution at the 2-position on Ado will prevent deamination, however the impact of multiple substitutions must be evaluated individually (as in the case of 2-amino-formycin A).

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*) in order to initiate a delineation of the mechanism of action of these compounds. Of the compounds tested, 2-aza-Ado, 2-fluoro-Ado, 2-chloro-Ado, 2-methyl-Ado, 2-fluoro-3-deaza-Ado, and 9-[β-D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine were the most promising in terms of antimycobacterial activity. 2-Methyl-Ado, 2-fluoro-3-deaza-Ado, and 9-[β-D-5-methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine 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*. 2-Aza-Ado and 2-chloro-Ado worked at least partially through Ado kinase as evidenced by their attenuated activity against SRICK1, and the activity of 2-fluoro-Ado was completely independent of Ado kinase.

Table 6.

Results of the structure-activity studies for *M. tuberculosis* and Human adenosine kinase

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
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	13 ± 2.5	+++			+
4	N^1-Ethyl-adenosine	< 1	+			
5	N^1-Bbenzyl-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	+			

Table 6 (Continued).

	Componnd name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	N^3-position					
21	3-Deaza-adenosine	1.4 ± 0.3	+		<1	-
22	2-Fluoro-3-deaza-adenosine	68 ± 14	+		<1	-
23	3-Fluoro-3-deaza-adenosine	16 ± 2	-		1 ± 0.8	+
24	2,3-Difluoro-3-deaza-adenosine	81 ± 11	-		6.1 ± 1	+
25	2,3-Difluoro-3-deaza-7-iso-adenosine	1 ± 0.7	-		5.2 ± 0.4	+
26	2-Fluoro-3-deaza-N^6-methyl-adenosine	<1	+		<1	+
27	3-Chloro-3-deaza-adenosine	<1	+		<1	-
	6-position					
28	N^6-Amino-adenosine	<2	+			
29	9-[β-D-Ribofuranosyl]-purine (purine riboside)	66 ± 11	+		3300 ± 1300	
30	6-Chloro-purine riboside	110 ± 13	++		2500 ± 1200	
31	6-Bromo-purine riboside	87 ± 14	++		2500 ± 390	
32	6-Iodo-purine riboside	<3	+			
33	6-Oxy-purine riboside (inosine)	<3	-			
34	6-Mercapto-purine riboside	<4	+			
35	6-Methyl-purine riboside	110 ± 6	++		980 ± 100	
36	6-Fluoromethyl-purine riboside	85 ± 6	++		2400 ± 720	
37	6-Methoxy-purine riboside	3 ± 1	+++			+
38	6-Methylmercapto-purine riboside	<1	+++			+
39	6-Cyclopentyloxy-purine riboside	<2	+++	0.15 m		+
40	6-Nitrobenzyl-mercapto-purine riboside	<1	+++			+
41	6-Benzyl-mercapto-purine riboside	<1	+++			+

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	2- and 6-positions					
42	2,N^6-Dimethyl-adenosine	2.4 ± 0.6	+			
43	2-Methyl-N^6-octyl-adenosine	solubility	++			
44	2-Methyl-N^6-benzoyl-adenosine	<0.2	+			
45	2-Chloro-purine riboside	1 ± 0.2	+			
46	2-Amino-6-oxypurine riboside (guanosine)	<1	+			
47	2-Methyl-6-oxypurine riboside	<2	+			
48	2-Chloro-6-methoxy-purine riboside	2.0 ± 0.3	++			
49	2,O^6-Dimethyl-inosine	1.2 ± 0.4	++			
50	2-Azido-O^6-methyl-inosine	1 ± 0.06	+++			+
51	2-Methyl-S^6-phenyl-6-mercaptopurine riboside	<2	+			
	7-position					
52	7-Deaza-adenosine (tubercidin)	<2	++			
53	7-Iodo-7-deaza-adenosine (iodotubercidin)	<2	+++	0.21c		++++
54	7-Cyano-7-deaza-adenosine (toyocamycin)	0.7 ± 0.2	+++			+++
55	7-Deaza-7-carboxamido-adenosine (sangivamycin)	<2	++			
	8-position					
56	8-Aza-adenosine	160 ± 50	++		910 ± 400	
57	8-Azido-adenosine	0.8 ± 0.06	+			
58	8-Chloro-adenosine	0.2 ± 0.06	+			
59	8-Bromo-adenosine	<1	+			
60	2-Fluoro-8-aza-adenosine	150 ± 11	++		220 ± 12	

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	6- and 8-positions					
61	6-Fluoro-8-amino-purine riboside	<1	+			
62	8-Chloro-purine riboside	<1	+			
63	8-Bromo-purine riboside	<4	+			
64	8-Hydroxy-purine riboside	<3	+			
65	8-Methoxy-purine riboside	<3	+			
66	8-Amino-purine riboside	<2	+			
67	8-Dimethylanimo-purine riboside	<2	+			
68	8-Mercapto-purine riboside	<1	+			
69	8-Methylmercapto-purine riboside	<1	-			
70	8-Azido-purine riboside	<1	+			
	8- and 9-positions					
71	8-Aza-9-deaza-adenosine (formycin A)	3800 ± 71	++	5.8 m	650 ± 20	+
72	2-Fluoro-formycin A	2200 ± 250	++		<1	+
73	2-Amino-formycin A	1800 ± 59	+		19 ± 6	-
	9-position					
74	9-Deaza-adenosine	<1	++			
75	6-Chloro-9-deaza-purine riboside	0.5 ± 0.07	++		3600 ± 200	+
76	2-Fluoro-6-chloro-9-deaza-purine riboside	0.6 ± 0.08	++		13 ± 1.3	+
	Multiple substitutions					
77	1-Deaza-2-amino-6-chloro-purine riboside	<2	+			

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i^c (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	Glycosidic bond position					
78	3-[β-D-Ribofuranosyl]-adenine	28 ± 2	++		268 ± 41	
79	7-[α-D-Ribofuranosyl]-adenine	<2	+			
80	8-Aza-8-[β-D-ribofuranosyl]-adenine	<1	+			
	4'-oxygen position					
81	2-Chloro-4'-thio-adenosine	<1	+			
82	Carbocyclic adenosine	1500 ± 230	+		860 ± 220	
83	2-Amino-carbocyclic-adenosine	17 ± 3	-		<2	
84	7-Deaza-carbocyclic-adenosine	<1	+			
85	2-Amino-8-aza-2'-deoxy-carbocyclic-adenosine	<6	-			
86	2-Amino-6-hydroxymethyl-8-aza-carbocyclic-adenosine	<2	-			
	2'-position					
87	2'-Deoxy-adenosine	13 ± 2	+		73 ± 11	
88	2'-O-Methyl-adenosine	<1	+			
89	2-Chloro-2'-deoxy-adenosine	<1	+			
90	2-Chloro-2'-deoxy-2'-fluoro-adenosine	<1	++			
91	2'-Deoxy-2,2'-difluoro-adenosine	29 ± 3	+		<1	
92	8-Aza-2'-deoxy-adenosine	<2	+			
93	9-[β-D-Arabinofuranosyl]-adenine (araA)	23 ± 2.3	-		6 ± 0	
94	2-Fluoro-araA	36 ± 3	+		<1	
95	2-Chloro-2'-deoxy-2'-fluoro-araA	<1	+			

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	2'-position (continued)					
96	1,4-Anhydro-2-deoxy-D-araA	<1	-			
97	1,4-Anhydro-2-deoxy-6-chloro-D-araA	<3	-			
	3'-position					
98	3'-Deoxy-adenosine (cordycepin)	<1	-			
99	3'-Deoxy-3'-amino-adenosine	0.2 ± 0.04	-			-
100	3'-Deoxy-3'-azido-adenosine	< 0.1	-			+
101	9-[β-D-Xylofuranosyl]-adenine	1 ± 0.1	-			
102	9-[β-D-Xylofuranosyl]-6-methyl-purine	<1	-			
103	9-[β-D-Xylofuranosyl]-6-oxy-8-aza-purine	<7	-			
	2'- and 3'-positions					
104	9-[β-D-2-Azido-2-deoxy-xylofuranosyl]-adenine	<1	-			
105	2-Chloro-2',3'-O-isopropylidene-adenosine	<1	-			
106	8-Aza-2',3'-O-isopropylidene-adenosine	<3	-			
	5'-position					
107	5'-Deoxy-adenosine	<1	++			
108	5'-Carboxamido-adenosine	<1	+			
109	5'-Amino-5'-deoxy-adenosine	YES	+++	0.8 c	<1	+++
110	9-[α-L-Lyxofuranosyl]-adenine	150 ± 24	+		360 ± 150	

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i[c] (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	5'-position (continued)					
111	9-[α-L-Lyxofuranosyl]-2-fluoro-adenine	130 ± 16	+		0.8 ± 0.2	
112	9-[β-D-5-Methyl-(*allo*)-ribofuranosyl]-2-fluoro-adenine	4 ± 0.3	++		<0.5	
113	9-[β-D-5-Methyl-(*talo*)-ribofuranosyl]-2-fluoro-adenine	1.5 ± 0.06	+		<0.7	
114	9-[β-D-5-Methyl-(*allo*)-ribofuranosyl]-6-methyl-purine	<2	++			
115	9-[β-D-5-Methyl-(*allo*)-ribofuranosyl]-6-methyl-purine	<1	+			
116	8,5'-(R)-Cycloadenosine	<1	-			
117	8-Aza-5'-deoxy-5'-methylsulfonyl-adenosine	<2	-			
	Multiple positions					
118	9-[β-L-Ribofuranosyl]-adenine	<1	-			
119	9-[α-D-Ribofuranosyl]-adenine	<1	+			
120	9-[α-D-Lyxofuranosyl]-adenine	<1	-			
121	7-[α-D-Arabinofuranosyl]-adenine	<2	+			
122	2',3',5'-Tri-O-acetyl-adenosine	<1	+			
123	Acyclo-adenosine	< 0.1	-			-
124	9-[2-[(2-Hydroxyethyl)-methylamino]-ethoxymethyl]-adenine	< 0.1	-			-

Table 6 (Continued).

	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i^c (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	Pyranoses					
125	9-[β-D-Ribopyranosyl]-adenine	<1	-			
126	9-[β-D-Allopyranosyl]-adenine	<1	-			
127	9-[β-D-Fructopyranosyl]-adenine	<1	-			
128	1-(Adenyl-9-yl)-1-doxy-β-D-glucopyranuramide	<1	-			
129	9-(2-Deoxy-β-D-*erythro*-pentopyranosyl)-adenine	<1	+			
130	9-(2-Deoxy-α-D-*erythro*-pentopyranosyl)-adenine	<1	-			
131	9-(3-Azido-3,4-dideoxy-β-D-*erythro*-pentopyranosyl)-adenine	<1	-			
132	9-[β-L-Galactopyranosyl]-adenine	<1	-			
133	9-[6-O-α-D-Galactopyranosyl-β-D-glucopyranosyl]-adenine	<1	-			
134	9-[α-D-Mannopyranosyl]-adenine	<1	-			
135	9-[α-D-Talopyranosyl]-adenine	<1	-			
136	6-(Adenyl-9-yl)-tetrahydropyran-2-methanol	<1	-			
	Other compounds					
137	Cytidine	<2	+			
138	Thymidine	<2	-			
139	Uridine	<2	+			

Table 6 (Continued).

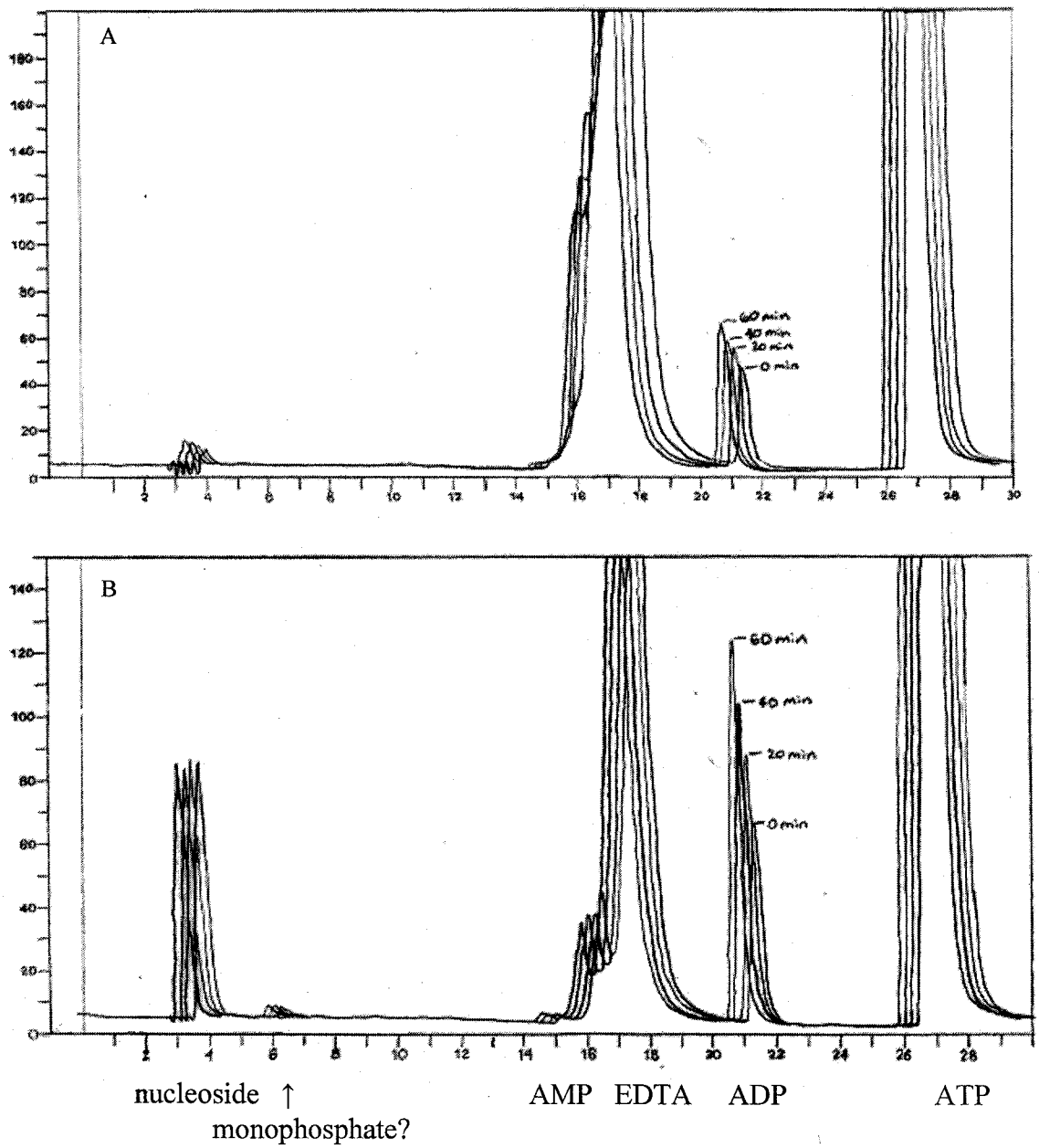
	Compound name	*M. tuberculosis* Specific activity[a] (nmol/mg-min)	Inhibition[b]	K_i^c (μM)	Human Specific activity (nmol/mg-min)	Inhibition[b]
	Other compounds (continued)					
140	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin)	3 ± 0.6	-			
141	2-β-D-Ribofuranosylthiazole-4-carboxamide (Tiazofurin)	<1	-			
142	2-β-D-Ribofuranosylselenazole-4-carboxamide (Selenazofurin)	<1	-			
143	2'-Deoxycoformycin (pentostatin)	<2	+			
144	6-Dimethylamino-9-[3-deoxy-3-(*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]-β-purine (puromycin)	<1	-			
145	9-(Cis-2-cyclopentyl)-purine	<1	-			
146	1-β-D-Ribofuranosyl-imidazo-[1,2-β]-pyrazole-7-carbonitrile	<1	-			
147	N-[4-(Benzyloxy)amidino]-1-β-D-ribofuranosyl imidazo-9-yl-formamide	<1	++			
148	4-Carboxamide-5-amino-1-β-D-arabinofuranosyl-imidazole	<2	-			
149	3-Amino-1-β-D-ribofuranosyl-triazolo-[5,1-c]-s-triazole	<1	+			

a. Specific activity was measured with 100 μM of each compound.

b. 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.

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

Figure 17.



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. 2 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.

Table 7.

MIC results with selected nucleosides

Compound	Activity[a] (nmol/mg-min)	MIC[b] (μg/ml) H37Ra	SRICK1	SRICK1::*adoK*
Ethambutol		1-10	1-10	1-10
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-Amino-Ado + dCF		1-10	>100	1-10
2-Methyl-Ado	74	1-10	>100	0.1-1
3-Deaza-Ado	1	>100	>100	>100
2-Flouro-3-deaza-Ado	63	1-10	>100	1-10
3-Fluoro-3-deaza-Ado	16	10-100	>100	10-100
3-Chloro-3-deaza-Ado	<0.2	>100	>100	>100
2,3-Difluoro-3-deaza-Ado	81	10-100	>100	10-100
2,3-Diflouro-3-deaza-7-iso-Ado	1	>100	>100	10-100
2-Fluoro-3-deaza-N^6-methyl-Ado	<0.1	>100	>100	10-100
8-aza-9-deaza-Ado (formycin A)	3808	>100	>100	>100
Formycin A + dCF		1-10	10-100	1-10
2-Fluoro-Formycin A	2200	10-100	10-100	10-100
2-Fluoro-Formycin A + dCF		10-100	10-100	10-100
2-Amino-Formycin A	1800	>100	>100	>100
2-Amino-Formycin A + dCF		1-10	>100	1-10
5'-Amino-5'-deoxy-Ado	ND[c]	>100	>100	>100
9-[β-D-5-Methyl-(*allo*)-ribofuranosyl]-Ade	4	1-10	>50	0.1-1
9-[α-L-Lyxofuranosyl]-2-fluoro-Ade	130	10-100	>100	10-100

a) Specific activity was measured as described in the materials and methods section using 100 μM of each compound.

b) MIC assays were performed at least twice for each compound.

c) ND - the specific activity could not be determined for this compound. Please see the results section for a full explanation.

Note: From "Structure-activity relationship for nucleoside analogs as inhibitors or substrates of adenosine kinase from Mycobacterium tuberculosis" by M.C. Long and W.B. Parker, 2006, *Biochemical Pharmacology*, 71 (12), p.1671-82. Copyright 2006 by Elsevier Inc. Reprinted with permission.

CHAPTER IV

DISCUSSION

Ado kinase was studied in an attempt to identify and biochemically characterize enzymes of the purine salvage pathway from *M. tuberculosis*. To the best of our knowledge, this work represents the first time that Ado kinase has been purified and characterized from a bacterium, and as such it provides insight into the evolution of this activity in these organisms. Throughout this work an effort has been made to include a comparison to human Ado kinase so that similarities and differences between the pathogen and host protein may be appreciated. Protein purification permitted identification and cloning of the gene which codes for Ado kinase. *M. tuberculosis* Ado kinase activity was dependent on Mg^{2+}, exhibited substrate inhibition, and demonstrated affinities for Ado and ATP that were similar to other Ado kinases. However, the enzyme that we identified exhibited some characteristics that are unique among Ado kinases, including its pH optimum profile, pI, quaternary structure, and lack of stimulation by P_i that were markedly different from other Ado kinases [56, 67, 82, 83]. In addition to providing a thorough biochemical characterization of a previously unstudied protein of the purine salvage pathway, this work yielded a model of the Ado binding domain of Ado kinase which should greatly advance the development of nucleoside analog antitubercular drugs.

Biochemical Characterization

All of the Ado kinases which have been characterized to date have been monomers. However, the quaternary structure of *M. tuberculosis* Ado kinase proved to be the most challenging to discern. This enzyme was initially thought to be a tetramer based on its elution from a gel filtration column, but subsequent analysis by light scattering photometry and analytical ultracentrifugation revealed that the native form of the enzyme was a dimer. Gel filtration chromatography separates proteins based on their size (Stoke's radius), and molecular weight is extrapolated from elution volume using the migration of globular proteins of known molecular weight as standards. Since it is not a direct measurement of molecular mass, molecular weight determination by gel filtration is prone to error. Light scattering photometry, sedimentation velocity, and sedimentation equilibrium analytical ultracentrifugation are considered to be direct measurements of molecular mass, and their results should be weighted accordingly. Even with buffer conditions similar to those for gel filtration, these methods uniformly indicated that the native form of the enzyme was a dimer. Therefore, we concluded that the native form of *M. tuberculosis* Ado kinase is a dimer.

Ado kinases from *M. tuberculosis* and human sources differed in physical parameters and, perhaps more interestingly, they differed in substrate and phosphate donor specificities (Table 4). *M. tuberculosis* and human Ado kinases are different enough to permit selective activation of Ado analogs like methyl-Ado. The MIC for methyl-Ado in *M. tuberculosis* (3 μg/ml) is 50 to 70 times lower than the concentration of methyl-Ado that inhibits 99% of growth in CEM cells (150 to 200 μg/ml). Studies of methyl-Ado phosphoryla-

tion by *M. tuberculosis* and human Ado kinases helped to explain the basis for the selective activation of methyl-Ado in *M. tuberculosis*. The K_m values for methyl-Ado demonstrated that methyl-Ado is a better substrate for *M. tuberculosis* Ado kinase than the human homolog (Table 4). These results suggest that the rate of phosphorylation of methyl-Ado by *M. tuberculosis* Ado kinase is at least partially responsible for the selectivity of this compound.

Initially the difference in methyl-Ado metabolism was not appreciated because assays for *M. tuberculosis* Ado kinase were performed without added KCl; under these conditions the K_m for methyl-Ado in *M. tuberculosis* was 710 μM, which was similar to the 960 μM K_m for methyl-Ado in human cells. After it was observed that K^+ had a stimulatory effect on Ado metabolism, the K_m for methyl-Ado was determined in the presence of KCl and found to have been significantly reduced to 79 μM. In the presence of KCl, the V_{max} for methyl-Ado increased from 60 to 180 nmol/mg-min. Methyl-Ado phosphorylation was stimulated by 300-fold in the presence of K^+. Since all living organisms maintain K^+ concentrations in the 100 to 500 mM range, stimulation by K^+ is physiologically relevant, and K_m and V_{max} values obtained in the presence K^+ more closely reflect the true physiological state of the enzyme than values obtained in the absence of K^+ [108].

The influence of K^+ on *M. tuberculosis* Ado kinase activity could not have been predicted based on its effect in other Ado kinases, although it is a characteristic of ribokinase [109]. Conflicting reports exist regarding the effect of K^+ on Ado phosphorylation by mammalian Ado kinase [57, 59, 83, 110]; however, the human enzyme requires K^+ in

order to phosphorylate deoxyadenosine [91, 110]. Studies with Ado kinase from *Leishmania donovani* have shown that KCl stimulates phosphorylation of formycin A, but not Ado [111], and emissions spectral analysis of Ado kinase from rabbit liver indicate that 0.9 mol of K^+ are bound per mol of Ado kinase [59]. Taken in context with the data from human and mammalian Ado kinase studies, it appears that K^+ may be bound to Ado kinases from various sources with little effect on phosphorylation of Ado. However, phosphorylation of other nucleosides and nucleoside analogs such as deoxyadenosine and formycin A may be stimulated in the presence of K^+. Unlike the other Ado kinases, in the *M. tuberculosis* enzyme Ado phosphorylation was also stimulated in the presence of K^+.

Another appreciable difference between human and *M. tuberculosis* Ado kinase lies in their respective preferences for phosphate donors. *M. tuberculosis* Ado kinase has demonstrated a preference for dGTP and GTP as phosphate donors with 4.7-times and 2.5-times the activity of ATP, respectively. Human Ado kinase has also demonstrated a preference for GTP and dGTP, although the preference is not as profound as that for *M. tuberculosis* Ado kinase (Figure 7). In the presence of GTP and dGTP, human Ado kinase exhibited at most 1.5-times the activity seen with ATP [92]. Since dGTP, GTP, and ATP exist at different intracellular concentrations, it is not yet possible to determine which of these phosphate donors is used intracellularly, or what affect these phosphate donors will have on Ado and methyl-Ado metabolism. Differences in the metabolism of methyl-Ado and a preference for dGTP and GTP as phosphate donors suggest that the active sites in *M. tuberculosis* and human Ado kinase may contain differences that can be exploited for drug development.

Analysis of the adoK *gene*

The amino acid sequence of *M. tuberculosis* Ado kinase was highly conserved among *Mycobacterium spp* with 86 to 100% homology; however, it was unique enough that no specific function could originally be assigned. In light of the identification of the function of the *Rv2202c* gene product as Ado kinase, we propose that the gene be re-named *adoK* in order to be consistent with the nomenclature of homologous genes. Human Ado kinase shares 50% or more sequence homology with Ado kinases from diverse sources, while it shared only 24% homology with *M. tuberculosis* Ado kinase. Based on primary structure, the *M. tuberculosis* enzyme could not be identified as Ado kinase, but was annotated as a member of the PfkB family of carbohydrate and purine nucleoside kinases. Although the percent of homology is still low (~35%), *M. tuberculosis* Ado kinase shares as much or more sequence homology with bacterial ribokinase and fructokinase as other Ado kinases.

According to amino acid sequence homology *M. tuberculosis* Ado kinase appears to be more evolutionarily related to other members of the PfkB family than other Ado kinases, indicating that Ado kinase activity may have arisen in *M. tuberculosis* as a result of convergent evolution. Indeed, phylogenetic analysis indicated that *M. tuberculosis* Ado kinase is more closely related to ribokinases than other Ado kinases (Figure 5). It is possible that *M. tuberculosis* Ado kinase represents a new class of bacterial Ado kinases within the PfkB family that is distinguished from other Ado kinases by its unique primary structure, quaternary structure, preference for reactants, and regulatory mechanisms. This work described the first bacterial Ado kinase to be characterized; however, several other

bacteria contain genes that share more than 50% sequence homology with Ado kinase from *M. tuberculosis*, that have not yet been assigned specific functions. Based on this homology, it is possible that this activity will be found in these other bacteria.

Why does M. tuberculosis have Ado kinase activity?

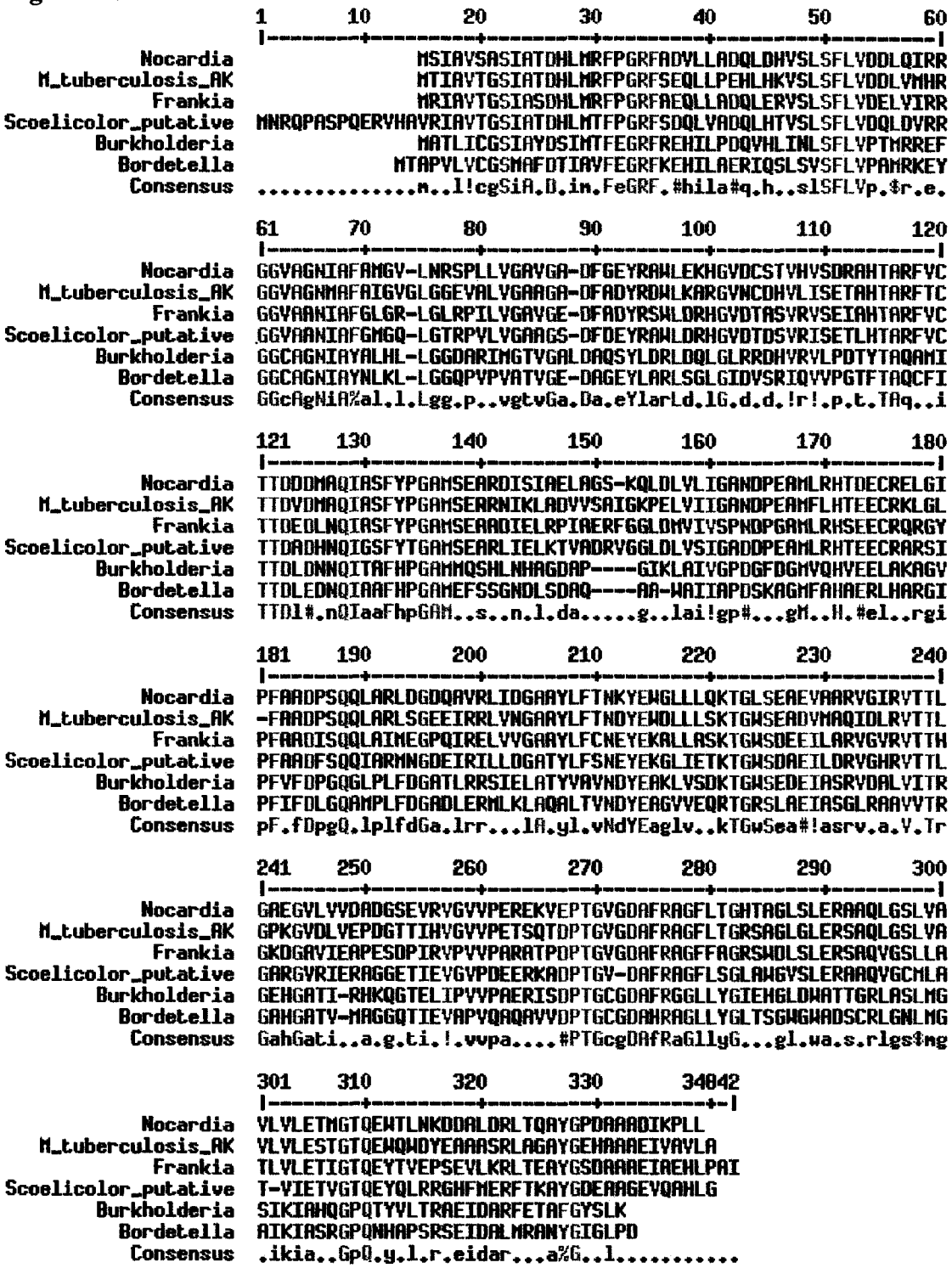
Identifying proteins which are essential for the survival of *M. tuberculosis* in vivo is a major goal for those who wish to develop antitubercular drugs. Each protein identified as being essential for growth represents a potential target for drug development. In an effort to identify all of the genes which are essential for the survival of *M. tuberculosis* during infection, two groups used transposon mutagenesis to create whole-genome mutant libraries [112-114]. One study analyzed 97% of the genes in the *M. tuberculosis* genome and identified 194 genes which are required for growth *in vivo* [113]. Of these, only 22 of these mutants were also defective for *in vitro* growth; therefore (lack of) growth *in vitro* is not predictive for (lack of) growth *in vivo*. Although both studies provided relatively thorough studies of the genome of *M. tuberculosis*, RV2202c (*AdoK*) was not evaluated in either study. We can only speculate on the role that Ado kinase serves for mycobacterial growth *in vivo*. One clue that Ado kinase may be important for in vivo growth comes from *Mycobacterium leprae*. *M. leprae* is considered to have a genome that is minimal for survival in vivo, yet this organism also conserves Ado kinase activity (100% sequence identity for Ado kinase from these bacteria). One school of thought holds that *M. leprae* has only conserved genes which are essential to its growth [112]; therefore, it follows that Ado kinase may be essential for the in vivo growth of some *Mycobacterium spp.*

At this time it is unclear why *M. tuberculosis* should contain Ado kinase while many other bacteria do not [37]. Ado kinase-deficient strains of *M. tuberculosis* and *M. smegmatis* survive well *in vitro* suggesting that Ado kinase is not essential in these conditions. *Mycobacterium spp* contain both *de novo* synthesis and salvage pathways for nucleotide metabolism (18, 30, 31, 32). It has been suggested that a limiting factor in the growth of *M. tuberculosis* is the slow rate of nucleic acid biosynthesis (33). Therefore it is possible that the purine salvage pathway in these bacteria has been specialized to allow the bacterium to take full advantage of nutrients available in their intracellular environment. Ado kinase activity is found in a wide variety of organisms including mammals, yeast, plants, and parasites; however, the paradigm has held that bacteria do not have Ado kinase activity [54, 55, 57-61, 67, 82, 111, 115-119]. In general, this may be true, but we are aware of two reports of Ado kinase activity in bacteria, *Chlamydia psittaci* and some *Mycobacterium* spp [26, 27, 75]. These bacteria share the traits of being intracellular pathogens which cause respiratory infections (*C. psittaci* causes respiratory infections in birds). However, there are many examples of intracellular bacteria that do not have Ado kinase activity, as in the case of *Listeria monocytogenes*. Therefore, this activity is not a widespread adaptation to intracellular life.

Several other bacteria have been identified as having Ado kinase or putative Ado kinase because they share sequence homology with Ado kinase from *M. tuberculosis*. *Streptomyces spp* and *Frankia spp* are among the bacteria that contain proteins which have the highest homology rates with *M. tuberculosis* Ado kinase (Figure 18); these bacteria are members of the Actinomycetales family. The most important pathogens that

have proteins which are identified as Ado kinases are *Burkholderia cepacia, Nocardia spp*, *Bordetella parapertussis* and *Bordetella bronchiseptica.* Along with *M. tuberculosis* and *C. psittaci*, these bacteria share the trait of causing lung infections. While *Nocardia, Bordetella,* and *Mycobacterium spp* belong to the order Actinomycetales, *Burkholderia spp* are Proteobacteria, diverging at the level of the phylum (*Mycobacterium* and others are Actinobacteria). *Burkholderia cepacia* causes severe lung infections in cystic fibrosis patients and *Bordetella spp* are the etiological agents of whooping cough, bronchitis, and a common cause of ear infection. These bacteria colonize cell surfaces and are not intracellular pathogens. *Nocardia* is an intracellular pathogen which is the etiologic agent of nocardiosis, a rare opportunistic infection that is very similar to tuberculosis. Although these respiratory pathogens are all likely to have Ado kinase activity, this function is not found in all respiratory pathogens, as evidenced by *Pseudomonas aeruginosa*, an opportunistic pathogen that commonly infects cystic fibrosis patients.

Figure 18.



Amino acid sequence alignment of bacterial Ado kinases. Amino acid sequence alignment of *M. tuberculosis* Ado kinase with predicted Ado kinases from *Nocardia spp.*, *Frankia spp.*, *Burkholderia cepacia*, and *Bordetella parapertussis*. Overall sequence ho-

mology was 67% with 24% sequence identity. Alignment was performed with Multalin software (http://prodes.toulouse.inra.fr/multalin/multalin.html).

It is possible that some respiratory pathogens developed Ado kinase activity in order to take advantage of the high levels of Ado found in lung tissue. Two major sources of intracellular Ado are the degradation of ATP and S-adenosylmethionine. ATP is degraded to Ado by ecto-5'-nucleotidases which sequentially break ATP down to its diphosphate and monophosphate forms before converting the nucleotide to Ado [37, 72, 120, 121]. The utilization of S-adenosylmethionine for methylation reactions results in the formation of S-adenosylhomocysteine, which is then hydrolyzed to homocysteine, with the release of Ado. The net result is an accumulation of Ado in lung tissue. Ado concentrations in normal human bronchoepithelial lining are much higher than circulating levels of Ado (60 μM and <0.2 μM, respectively) [122]. Ado undergoes both equilibrative and concentrative transport into cells where it is rapidly metabolized to Ino or AMP. Under steady-state conditions, in the presence of such high concentrations of Ado, it is possible that cells in the lungs are more Ado-rich than cells in other tissues.

Ado is produced at high levels in the lung in response to lung hypoxia, injury, infection, and inflammation where it plays a role in the pathology of asthma and chronic obstructive pulmonary disease (COPD) [122-124]. In asthma patients, Ado concentrations reach almost 200 μM in bronchoepithelial fluids, largely due to mast cell stimulation and degranulation [122, 123]. An animal model of COPD was created by knocking out Ado deaminase activity in mice. These Ado deaminase-knockouts had levels of Ado

in their lungs which was similar to that seen with COPD and asthma, and these increased levels of Ado resulted in tissue remodeling similar to what is seen with COPD [123, 124]. In the past, levels of Ado that are available during *M. tuberculosis* infection were extrapolated from studies of circulating levels of Ado and Ado in different tissues (<0.1 μM) [28, 121]. However, the groups that performed these studies were never specifically searching for Ado levels in lung tissue of tuberculosis patients. Although specific information was not available about Ado levels in bronchoepithelial fluids of tuberculosis patients, it is possible that Ado levels in this tissue rise to levels seen with other chronic inflammatory disease states.

The fact that *Mycobacterium spp* have *de novo* synthesis argues against Ado kinase being essential. However, the rate of nucleic acid biosynthesis is slow enough to be growth-limiting in slow growing mycobacteria [28]. It is possible that *Mycobacterium spp*, including *M. tuberculosis*, evolved Ado kinase activity as a means to supplement their slow rate of nucleic acid synthesis. By salvaging preformed purine bases, *M. tuberculosis* can take advantage of the Ado-rich environment in the human lung. Arguments for the essentiality of Ado kinase are bolstered by the complete conservation of this enzyme in *Mycobacterium leprae*. However, in the absence of an *adoK*⁻ mutant of *M. tuberculosis*, it is not possible to definitively determine whether or not Ado kinase activity is essential for the growth of this organism *in vivo*.

Structure-activity relationship.

Ado kinase from *M. tuberculosis* 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)** [32, 51, 52]. The goal of this study was to identify structural modifications to 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 5-fold. For competitive inhibitors, the relative affinity of each compound may be determined by applying the relationship of Cheng and Prusoff where $(I_{50})_1/(I_{50})_2 = (K_i)_1/(K_i)_2$ [125]. 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 an estimate of the affinity of compounds that inhibited phosphorylation of 0.1 μM Ado at a level of 1 μM. Each 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 were both substrates and inhibitors, 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 accurate for compounds which inhibited at 10 or 1 μM (ie had greater affinity and lower K_m) than for compounds which inhibited only at 100 μM. For compounds which were poor inhibitors (ie inhibited Ado phosphorylation by 20% at 100 μM), the velocity measured at 100 μM of the compound is an underestimate of the actual V_{max}. Furthermore, some of the most

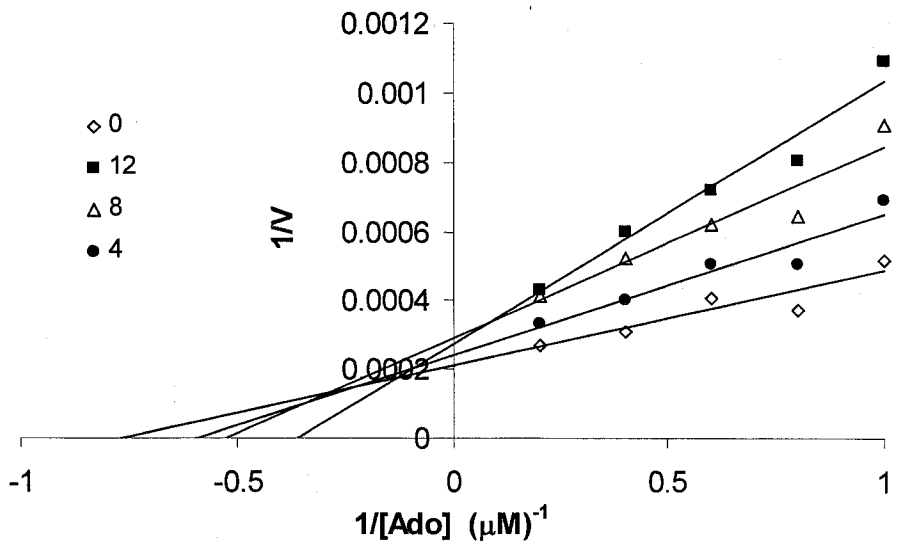
potent inhibitors were not substrates at all, such as the 7-deaza-Ado series **(47-50)** and some 6-substituted purine ribosides **(32-36)**. Results such as this indicate that these compounds would have a low K_m and low V_{max}. Review of the results in this manner can provide an estimate of the Michaelis-Menton 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 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 (Figure 19). 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 [79, 80]. 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 [95, 126].

Drug development efforts are focused on using Ado kinase as 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 compound (Tables 6 and 7). As long as the phosphorylated product is potent enough to 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.

Figure 19.



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; Δ- 8 μM, R = 0.96; and ♦-12 μM, R = 0.99.

Note: From "Structure-activity relationship for nucleoside analogs as inhibitors or substrates of adenosine kinase from Mycobacterium tuberculosis" by M.C. Long and W.B. Parker, 2006, *Biochemical Pharmacology*, 71 (12), p.1671-82. Copyright 2006 by Elsevier Inc. Reprinted with permission.

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. One important observation is that for compounds with multiple substitutions, inclusion of a small, exocyclic substitution at the 2-position (ie a fluoro, chloro, amino, or methyl group) improves the selectivity of a compound for *M. tuberculosis* Ado kinase. This holds true even though the single substitution, 2-fluoro-Ado **(10),** is a better substrate for human than *M. tuberculosis* Ado kinase. Substitutions at the 2-position do not have a predictable effect on the specific activity of the compounds in *M. tuberculosis* Ado kinase, some have increased activity, decreased activity, or similar activity to their parent compounds (Table 8). The main factor that tips the balance of selective activity in favor of the tuberculosis enzyme is that inclusion of a substitution at the 2-position reliably reduced the substrate activity of the compound in human Ado kinase (Table 8). Another consideration for inclusion of an exocyclic 2-substitution is that, with the exception of an exocyclic amino group, this type of modification reduces the likelihood that the compound will be a substrate for Ado deaminase.

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 [79, 86, 96, 97, 127]. 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 (Figure 20) in the 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 and ribose moieties that may be useful for development of nucleoside analog antitubercular compounds (Appendix A).

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.

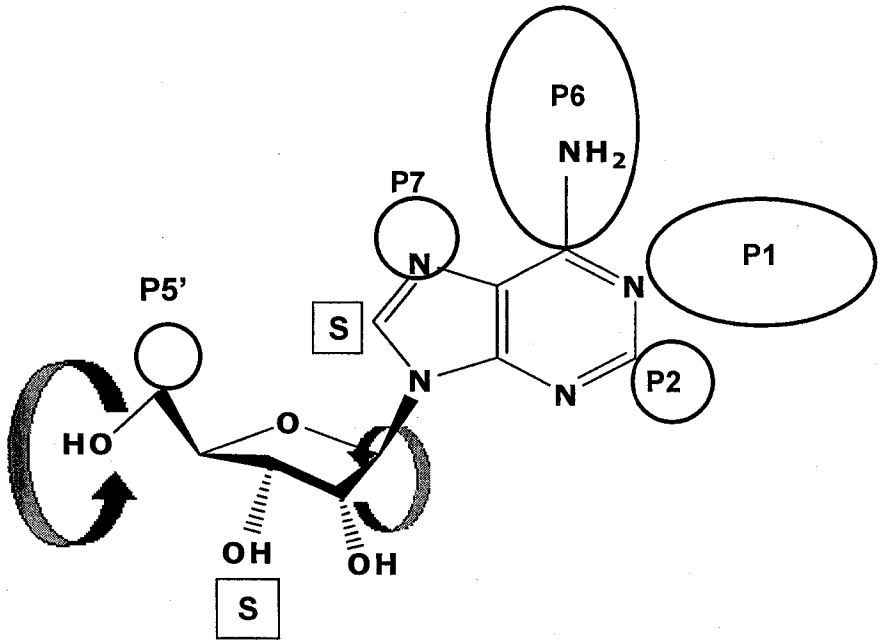
Table 8.

Effect of exocyclic 2-substituted Ado analogs on enzyme selectivity

Compound	Ado kinase Activity (% of Ado control) *M. tuberculosis*	Human	Ratio[a]
Single substitutions:			
Adenosine	100	100	1
2-Fluoro-Ado	52	75	0.7
2-Chloro-Ado	12	0.7	17
2-Amino-Ado	6	5	1.2
2-Methyl-Ado	1.9	0.2	9.5
Effect of multiple substitutions:			
3-deaza-Ado	0.03	<0.04	>0.8
2-Fluoro-3-deaza-Ado	1.7	<0.04	42
8-aza-Ado	4	38	0.1
2-Fluoro-8-aza-Ado	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	95	25	3.8
2-Fluoro-formycin A	55	<0.04	1375
2-Amino-formycin A	45	0.6	75

a. The 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.

Figure 20.



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 and 3'-positions denotes a steric blockade for exocyclic substitutions at these sites. P5' occurs at the site of catalytic activity. This region can accommodate an extra methyl group on the 5'-C. Arrows at the 2' and 5'-positions denote the ability to bind compounds with rotation around the 2'-C and 4'-C, as evidenced by the activity of araA **(93)** and 9-[α-L-Lyxofuranosyl]-adenine **(110)** respectively.

Note: From "Structure-activity relationship for nucleoside analogs as inhibitors or substrates of adenosine kinase from Mycobacterium tuberculosis" by M.C. Long and W.B. Parker, 2006, *Biochemical Pharmacology*, 71 (12), p.1671-82. Copyright 2006 by Elsevier Inc. Reprinted with permission.

Future directions

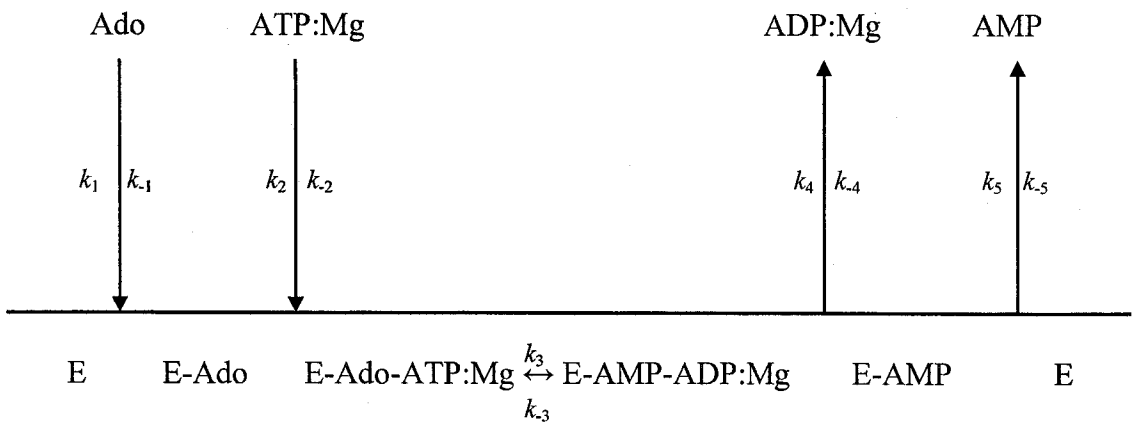
This work represents the first step in targeting uncharacterized proteins involved in purine salvage in *M. tuberculosis* for identification and characterization. Other enzymes that are currently under investigation are guanosine kinase and Ado hydrolase. Ado kinase was studied because of its role in selectively activating methyl-Ado to a toxic metabolite. Biochemical characterization revealed positions on Ado which can be modified in order to create substrates that will be specific for *M. tuberculosis* Ado kinase, and not human. In the absence of the drug development aspect of this project, the purification and characterization of the enzyme can stand on its own, as it identified a protein that is unique among bacteria. For this reason there are several major directions for future research related to Ado kinase from *M. tuberculosis.* Further studies of the protein should include crystallography and determination of reaction kinetics. Drug development efforts should involve synthesis of new compounds, testing for substrate and inhibitor activity, and MIC analysis. Furthermore, efforts should be made to determine if Ado kinase is essential for the growth and survival of *M. tuberculosis* in vivo; this would begin to answer the question as to why *M. tuberculosis* has this activity that is most uncommon for a bacterium.

Enzymatic analysis. A great deal is known about Ado kinases from a wide variety of sources. However, this protein is evolutionarily distinct from other known Ado kinases. Although this work provided an initial biochemical characterization of Ado kinase from *M. tuberculosis*, it opened up new avenues for analysis of this protein. One avenue of study is the determination of the mechanism of catalysis for this protein, similar to the

work performed with human Ado kinase which determined the mechanism for the binding and release of reactants and substrates to be an Ordered Bi Bi mechanism (Figure 21) [58, 92, 128]. It would be interesting to compare the kinetic mechanisms and modes of regulation, such as the requirements for pentavalent ions and modulation of activity by reactants and products for these two proteins.

Determination of a high-resolution crystal structure will also be an important goal of future work. There currently exists a low-resolution structure for this protein; however, it is lacking details about the active site that will be helpful for confirming kinetic mechanisms, identifying the active site, and providing a high-resolution picture of the Ado-binding domain that will aid the further development of antitubercular nucleoside analogs. The current model of *M. tuberculosis* Ado kinase was able to confirm the similarity that this protein bears to ribokinase, in particular it verified our finding of a dimeric quaternary structure (Appendix B). It will be of interest to co-crystallize this protein with some of the compounds that have proven to be good substrates and good inhibitors in order to identify interactions which are essential for these activities. Combining the SAR results with a high-resolution crystal structure will contribute to future drug development with this enzyme by permitting a 3D-quantitative SAR. Once we have a clear picture of the active site, the compounds that were used in the SAR can be modeled *in silico* in order to determine the necessary components of a pharmacophore for this enzyme.

Figure 21.



Schematic of an Ordered Bi Bi Reaction. The reaction mechanism depicted here is adapted from reports for human Ado kinase [58, 92, 128].

Drug development. The structure-activity relationship provided a rough idea of some of the topographic features of the active site, identifying hydrophobic pockets, sites of steric hindrance, and sites that are open to modification for selective substrates. This work should be continued. Based on the results of the SAR, we have proposed the synthesis of 32 Ado analogs (Appendix C) for future evaluation as substrates and inhibitors of Ado kinase. Furthermore, since substrate activity does not correlate directly with antitubercular activity, MIC evaluations should be performed with all of the compounds evaluated in this study. Initial MIC analysis can be performed at a single concentration (ie 12.5 μg/ml, similar to concentrations that the TAACF uses). For compounds that demonstrate antitubercular activity, MIC studies should then be performed with *M. tuberculosis* strains H37Ra, SRICK1, and SRICK1::*adoK* in order to determine if the mechanism of action works through Ado kinase. A continuation of the *in vitro* determination of antitubercular activity should involve determination of efficacy of each compound in a macrophage model of infection. This model would simulate infection *in vivo* better than screens in liquid culture. This would also permit simultaneous determination of cytotoxicity in uninfected control cultures. Since antibiotic-resistant strains of *M. tuberculosis* are available, determination of MIC in these strains would provide a more complete in vitro evaluation of the activity of these compounds. Much of the work that is proposed here can be accomplished through the mechanism of the TAACF with the exception of MIC studies in Ado kinase-deficient and complemented strains of *M. tuberculosis*.

Another aspect of the development of Ado analogs as antitubercular drugs that should not be neglected is the evaluation of the interaction of these compounds with Ado

receptors. Purinergic receptors are widely distributed throughout the body and many of them have been characterized [68-70, 72]. These receptors bind Ado and ATP which play important, yet opposing roles as extracellular signaling molecules; ATP is considered to be excitatory, while Ado is inhibitory [70, 72]. It is thought that these opposing effects are by design since the conversion of ATP to Ado serves as a control mechanism for ATP signaling. ATP interacts with P2 -purinergic receptors which serve as fast excitatory neurotransmitters and play an important role in nociception and platelet aggregation [70, 72]. Whereas, Ado interacts with P1-purinergic receptors, a group of G-coupled protein receptors that play a protective role in ischemia, are antiinflammatory, improve vasodilation, and aid in the dissolution of aggregated platelets [70, 72]. Ado receptors are highly concentrated in brain tissue and Ado has profound sedative and hypnotic effects due to interaction with these receptors [72]. It is possible that the interaction of methyl-Ado with these receptors was responsible for its sleep-inducing side effect. This side effect may not occur in humans because the ligand-specificity of human and mouse Ado receptors is known to differ. Future studies arising from this work should include the evaluation of any potential drugs as ligands for human Ado receptors.

Although Ado kinase is the only enzyme which has been identified as being involved in the mechanism of action of methyl-Ado, it is likely that other enzymes that are also involved. The current status of our knowledge of the mechanism of action of this compound is currently limited. One future goal may be to identify other enzymes involved in the mechanism of action of 2-methyl-Ado so that future drug development efforts can focus on the downstream target(s) instead of only activation by Ado kinase.

Transposon mutagenesis may be used in order to identify other genes which may be involved in the mechanism of action of methyl-Ado.

***Creation of an Ado kinase knockout in order to determine if Ado kinase is essential for the growth and survival of* M. tuberculosis in vivo**. One question that looms large over this work is the question of why *M. tuberculosis* has Ado kinase activity at all. This is a difficult question to answer; however, one can create a hypothesis that is testable such as, *M. tuberculosis* contains Ado kinase activity because it is necessary for this bacterium to grow *in vivo*. The only way to test this hypothesis is to create an Ado kinase knockout strain of *M. tuberculosis* and assess its growth in an animal model of infection. There are two ways to knockout Ado kinase activity, either eliminate all, or a portion of the *adoK* gene (unmarked deletion), or interrupt the gene with an intervening sequence which will render it non-functional (marked deletion). A second strain should be created in which the knockout strain is complemented with the *adoK* gene. This complemented strain should bear either a single-copy plasmid or have the *adoK* gene stably integrated back into genomic DNA so that Ado kinase activity is expressed at native levels.

Once these strains are created, growth curves should be created in liquid culture and in macrophage in order to begin to determine if the *adoK*-deficient strain is in any way deficient in growth *in vitro*. As mentioned previously, growth in vitro is not predictive for growth in vivo; therefore, these experiments should not preempt determination of growth in an animal model [113]. These strains of bacteria will prove useful for future determination of MIC levels in wild-type, Ado kinase-deficient, and complemented

strains of *M. tuberculosis*. They will likely replace the existing strains which are created from an avirulent parent strain.

In summary, identification and characterization of Ado kinase from *M. tuberculosis* has opened up multiple avenues for future research. Although each of the research projects proposed herein can stand alone, they bear more power when integrated together. For instance, if Ado kinase is demonstrated to be beneficial for growth in vivo, this result will begin to shed light on the origin of this activity in *M. tuberculosis* and it will extend the drug development aspect of this project, since the SAR has provided multiple selective and potent inhibitors of *M. tuberculosis* Ado kinase. Furthermore, crystallization of the protein will undoubtedly shed light on the binding mechanism of the protein and reveal interactions which are essential for ligands to bind to the active site. Until then, we can continue to probe the active site chemically by synthesizing new Ado analogs as a continuation of the SAR. Each of these research components serves to advance the development of Ado analogs as antitubercular drugs.

Conclusion

Ado kinase from *M. tuberculosis* proved to be unique among known Ado kinases, being more similar to ribokinase in terms of primary and quaternary structures and stimulation by K^+. Indeed, Ado kinase from *M. tuberculosis* is different enough from its human homolog to permit the selective phosphorylation of methyl-Ado. Ado kinase activity had been previously reported in some *Mycobacterium spp* and *Chlamydia psittaci*, but the protein had never been characterized. Identification of Rv2202c (*adoK*) as the gene that

codes for Ado kinase led to the identification of this gene in several other bacteria, including some important lung pathogens. As more bacterial genomes become available, it is possible that similar genes will be found elsewhere. Identification of the *adoK* gene provided the information necessary to create an *adoK*⁻ strain of *M. tuberculosis* in order to determine if this activity is necessary for the growth of *M. tuberculosis in vivo*. If Ado kinase is necessary for growth and survival of this pathogen in vivo, then Ado kinase itself will become the target for development of selective inhibitors.

This work laid the foundation for future development of nucleoside analogs as antitubercular prodrugs which work through Ado kinase. The advantage of this class of drugs is that they should be useful against drug-resistant strains of *M. tuberculosis* because they are unlikely to share a mechanism of action with current antitubercular drugs. The SAR permitted identification of several sites on Ado which may be modified to permit selective phosphorylation of Ado analogs in *M. tuberculosis*. A number of new compounds have been proposed for synthesis based on this work (Appendix C). Once a high-resolution crystal structure is acquired, the SAR results can be re-analyzed in context of the Ado binding domain to determine the specific requirements of a pharmacophore for this protein. All of the elements are in place for continued development of nucleoside analogs as antitubercular agents.

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APPENDIX A

Structure-Activity Relationship Summary of Results

Appendix A.

Structure-Activity Relationship 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* (2-aza-Ado, **9**). 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. Exocyclic substitutions at this position increased the selectivity for *M. tuberculosis* Ado kinase, even a 2-fluoro group (although 2-fluoro-Ado **(10)** was more active in human Ado kinase). In the future, we recommend that compounds synthesized for testing as substrates for *M. tuber*culosis Ado kinase have a small, exocyclic substitution at the 2-position, such as a fluoro, chloro, methyl, or amino group, in order to increase selectivity for this enzyme and circumvent deamination.
N^3	The endocyclic *N* was very important for substrate activity, perhaps acting as a hydrogen bond acceptor. Exocyclic substitutions at the 3-position may be limited

by size. 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)**. Other 2-substituted 3-deaza-Ado analogs should be investigated in the 3-deaza-Ado series because they were promising for selectivity (see Appendix B for suggestions).

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 (Formycin A, **71**) 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. When coupled with substitutions at the 2-position, the selectivity for formycin A as a substrate increases.

2' A trans-2'-hydroxyl group was preferred, although a cis-2'-hydroxyl group (araA, **93**) and 2'-deoxy-Ado **(87)** were also substrates at very low levels. Compounds in this class were poor inhibitors unless they also had a second substitution at the 2-position.

3' A trans-3'-hydroxyl group was preferred although a cis-3'-hydroxyl group (9-[β-D-xylofuranosyl]-adenine, **101**) had measurable albeit low activity. Substitutions 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 **(82)** was a good substrate for both human and *M. tuberculosis* AKs. The selectivity for carbocyclic-Ado analogs can be improved with the addition of an exocyclic substitution at the 2-position.

5' Substitutions to the 5-position were poorly tolerated. The best substrates in this category were 9-[α-L-lyxofuranosyl]-adenine **(110)** and 2-F-9-[α-L-lyxofuranosyl]-adenine **(111)**, both maintained about 3% of the activity of Ado. Although it has yet to be confirmed, 5'-amino-5'-deoxy-Ado **(109)** appeared to be

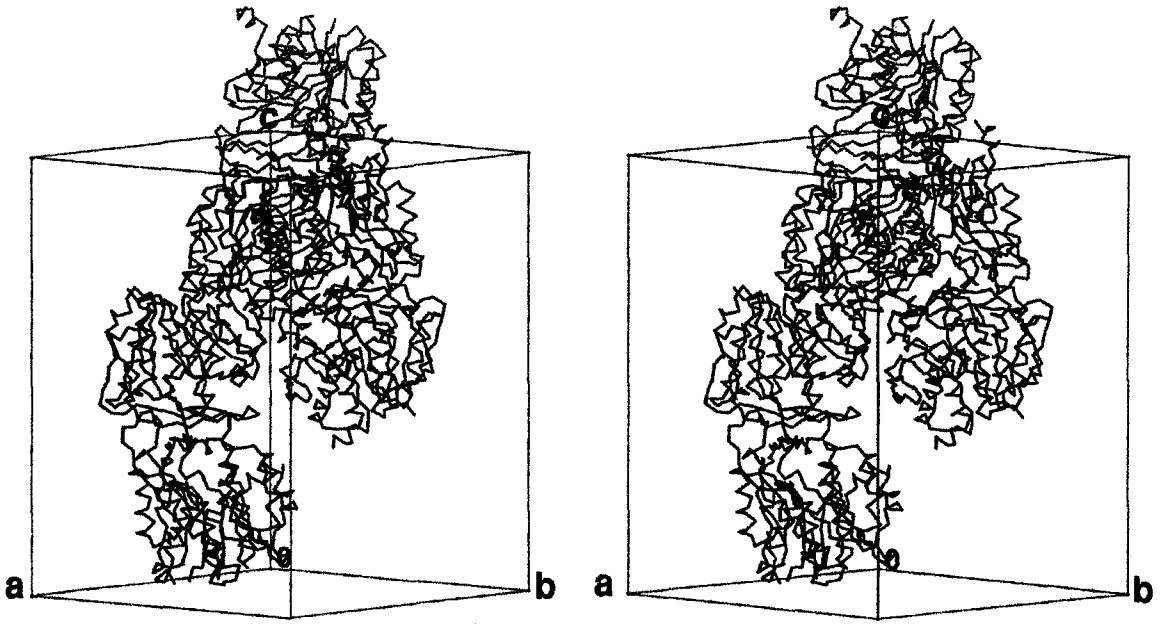
a substrate for this enzyme. This compound was also the best inhibitor of all of the substitutions to the ribose moiety.

APPENDIX B

The Current Model of the Ado kinase Crystal Structure

Appendix B.

The Current Model of the Ado kinase Crystal Structure



Stereoview of the molecular packing of the unit cell for an *M. tuberculosis* Ado kinase crystal. Three dimers are shown in the Cα backbone structure. The monomers of each molecular dimer are related by twofold crystallographic symmetry (as highlighted in red and green). This figure was generated by MOLSCRIPT (Kraulis, 1991).

Note: From "Overexpression, purification, and crystallographic analysis of a unique adenosine kinase from *Mycobacterium tuberculosis*" by Wang, Y., Long, M.C., Ranganathan, S., Escuyer, V., Parker, W.B., and Li, R., 2005, *Acta Crystallographica*, F61, p.553-7. Copyright 2005 by International Union of Crystallography, reprinted with permission.

APPENDIX C

Compounds proposed for testing as Ado kinase substrates and inhibitors

Appendix C.

Compounds proposed for testing as Ado kinase substrates and inhibitors

3-deaza-Ado series:
- 2-chloro-3-deaza-Ado
- 2-methyl-3-deaza-Ado

6-methyl-purine riboside series:
- 2-fluoro-6-methyl-purine riboside
- 2-chloro-6-methyl-purine riboside
- 2-amino-6-methyl-purine riboside

8-aza-Ado series:
- 2-chloro-8-aza-Ado
- 2-amino-8-aza-Ado
- 2-methyl-8-aza-Ado

8-aza-9-deaza-Ado (Formycin A) series:
- 2-chloro-formycin A
- 2-methyl-formycin A
- 2-aza-formycin A

Carbocyclic-Ado series:
- 2-fluoro-carbocyclic-Ado
- 2-chloro-carbocyclic-Ado
- 2-aza-carbocyclic-Ado
- 2-fluoro-8-aza-carbocyclic-Ado
- 8-aza-9-deaza-carbocyclic-Ado

2'-hydroxyl series:
- 2'-amino-Ado
- 2-fluoro-8-aza-9-deaza-9-[β-D-arabinofuranosyl]-adenine
- 2-aza-9-[β-D-arabinofuranosyl]-adenine

5'-carbon series:
- 2-amino-9-[α-L-lyxofuranosyl]-adenine
- 2-methyl-9-[α-L-lyxofuranosyl]-adenine
- 2-aza-9-[α-L-lyxofuranosyl]-adenine
- 8-aza-9-deaza-9-[α-L-lyxofuranosyl]-adenine
- Eliminate the 5'-carbon, replace with a hydroxyl group

2', 3', or 4'-carbon series:
- Attempt to substitute an oxygen or nitrogen atom at these positions within the ribose structure. Try in combination with an adenine base first.

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UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Mary Catherine Long

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Title of Dissertation Adenosine Kinase From *Mycobacterium tuberculosis*

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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