The Purine Salvage Pathway as a Target for Structure-Based Drug Design in the Intracellular Parasites *Leishmania donovani, Mycobacterium avium* and *Mycobacterium tuberculosis*

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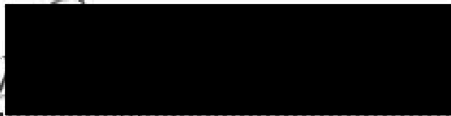

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TABLE OF CONTENTS

ABSTRACT ix

INTRODUCTION 1

A. Structure-based Drug Design 1

1. Approaches to Drug Design 3
2. Purine Salvage Pathway as a Therapeutic Target 21

B. *Leishmania donovani* 37

1. Life cycle 38
2. Visceral Leishmaniasis 46
3. Treatment and Control 48
4. Drug Resistance and Future Outlook 52

C. *Mycobacterium tuberculosis* 54

1. Life cycle 58
2. Tuberculosis 68
3. Treatment and Control 68
4. Drug Resistance and Future Outlook 74

D. *Mycobacterium avium* 79

1. An opportunistic pathogen in immunosuppressed patients 79

2. Treatment and Control....................80

3. Drug Resistance and Future Outlook....................81

THESIS RATIONALE....................83

SPECIFIC AIMS....................85

MATERIALS AND METHODS....................86

RESULTS....................96

DISCUSSION....................160

CONCLUSION....................176

REFERENCES....................177

APPENDIX....................196

CATALOG OF FIGURES

Figure 1	Comparison of Metabolic Pathways	6
Figure 2	*Mycobacterium tuberculosis* Cell Envelope	15
Figure 3	Purine Salvage Pathway	23
Figure 4	The Purines	25
Figure 5	Phosphoribosylation Reaction	27
Figure 6	Comparison of HGPRTs	31
Figure 7	The Pyrazolopyrimidines	33
Figure 8	Comparative Allopurinol Metabolism	35
Figure 9	Old and New World *Leishmania sp.*	39
Figure 10	World Distribution of *L. donovani*	41
Figure 11	Life Cycle of *L. donovani*	43
Figure 12	Receptor Ligand Mediated Phagocytosis of *M. tuberculosis*	60
Figure 13	Molecular Target of Drug Resistance in *M. tuberculosis*	77
Figure 14	Alignment of XPRT and HGPRT from *L. donovani*	97
Figure 15	Southern Analysis of a *L. donovani* cosmid clone	99
Figure 16	Cosmid Map of Clone Containing *xprt*	101

Figure 17	Nucleotide sequence of the *L. donovani xprt* and deduced amino acid sequence	103
Figure 18	Southern blot analysis of the *L. donovani* DI700 *xprt* locus	106
Figure 19	Complementation of the bacterial *g(x)prt* mutation	108
Figure 20	Overproduction and purification of recombinant XPRT	111
Figure 21	Hanes analysis of recombinant *L. donovani* XPRT	113
Figure 22	Southern blot analysis of *M. avium* and *M. tuberculosis* chromosomal DNA	117
Figure 23	Southern analysis of the *M. tuberculosis hgprt* cosmid	119
Figure 24	Nucleotide sequence of *M. avium hgprt* and deduced amino acid sequence	122
Figure 25	Nucleotide sequence of *M. tuberculosis hgprt* and deduced amino acid sequence	124
Figure 26	Alignment of *M. avium* and *M. tuberculosis* '5 upstream *hgprt* region	127
Figure 27	Complementation of *hpt* and *gpt* mutations in Sφ609 *E. coli* with *M. avium* and *M. tuberculosis hgprt*	129
Figure 28	Multiple sequence alignment of various HGPRT's	132
Figure 29	Comparison of HGPRT amino acid sequence	134

Figure 30 Hanes Analysis of *M. tuberculosis* HGPRT (hypoxanthine) 137

Figure 31 Hanes Analysis of *M. tuberculosis* HGPRT (guanine) 139

Figure 32 Isoelectric Focusing of Purified *M. avium* and *M. tuberculosis* HGPRTs 141

Figure 33 Magnesium Effect on HGPRT 143

Figure 34 Cation Effect on HGPRT 145

Figure 35 Effect of pH on HGPRT 147

Figure 36 Crystallization of *M. tuberculosis* HGPRT 150

Figure 37 Primary *in vitro* Drug Screen of Purine Analogs Against *M. avium* and *M. tuberculosis* 153

Figure 38 Secondary *in vitro* Drug Screen of Purine Analogs Against *M. avium* and *M. tuberculosis* 155

Figure 39 LineWeaver-Burk Analysis of *M. tuberculosis* HGPRT 158

Figure 40 Purification of *M. avium* and *M. tuberculosis* HGPRTs 202

Figure 41 Purification of *M. tuberculosis* HGPRT 204

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Warm mud squished up between my toes. I steadied my B-B gun. Blamm! Instantly the frog died and I began my quest to understand it's body parts by a simple dissection with the prized knife my father had given me. As I sit here writing my thesis and contemplating how I transformed from a somewhat uncivilized Tom-boy to a researcher who utilizes more advanced technology for discovery, I realize I have a plethora of people to thank for their contributions to my success. At the top of the list are my parents who refrained from telling me that my second grade teacher had informed them to demand that I NEVER again, under any circumstances, bring isopropanol preserved frog parts for show-and-tell. My mother, who most probably would have liked to see her daughter in dresses and pretty shoes, endured unruly hair, cutoffs and most of the time bare feet, hence the thick callouses as I searched through nature's beauty finding rocks, bugs, plants and leaves to identify and preserve them. Never once did she ask me to remove my specimens from her books or freezer.

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ABSTRACT

The purine salvage pathway has stimulated considerable therapeutic interest among scientists and clinicians alike for its unique features found in different organisms, especially the ability to initiate metabolism of cytotoxic analogs of naturally occurring purines. To validate xanthine phosphoribosyltransferase (XPRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) as potential therapeutic targets, the *xprt* gene from *Leishmania donovani* and the *hgprt* genes from *Mycobacterium avium* and *Mycobacterium tuberculosis* were cloned and sequenced.

L. donovani xprt was cloned utilizing functional complementation of a cDNA library in purine auxotrophic Sϕ609 *E. coli*, genetically deficient in the bacterial phosphoribosyltransferase counterparts. The *xprt* cDNA was then used as a hybridization probe to clone the genomic version of *xprt* from a cosmid library. Southern analysis revealed that the *xprt* gene exists as a single copy and is located within three kilobases downstream of the *hgprt* gene.

A 174 bp fragment of the *M. avium hgprt* gene was amplified by the polymerase chain reaction (PCR) using degenerate oligonucleotide primers constructed to two domains that are highly conserved on HGPRT proteins among genetically diverse organisms. The PCR fragment was then used as a probe to isolate the full length *M. avium* and *M. tuberculosis hgprt* genes from

corresponding genomic libraries.

Sequence analysis of positive clones exhibited open reading frames of 723, 697 and 694 nucleotides encoding polypeptides of 27.0, 24.0, and 23.9 kD for *L. donovani xprt, M. avium hgprt* and *M. tuberculosis hgprt* respectively. Alignment of the HGPRT sequences with the human counterpart indicated a 26% identity for both *M. avium* and *M. tubercuolsis*. As humans do not have an *xprt* gene, comparison of XPRT was made to the *L. donovani* HGPRT protein. A 26% identity and 50% homology was predicted, suggesting a primordial gene duplication event given the extent of homology that exists between the two genes and their close proximity.

The *xprt* and *hgprt* genes were ligated into the prokaryotic expression vector pBAce. Transfected purine auxotrophic Sϕ609 *E. coli* cells grown in low phosphate induction media supplemented with xanthine or hypoxanthine, as appropriate, overproduced the individual recombinant proteins. Purification of the XPRT and HGPRT's to apparent homogeneity via differential ammonium sulfate precipitation followed by gel filtration allowed kinetic analysis. Alternatively, small amounts of mycobacterial HGPRT could also be rapidly purified by affinity GTP-agarose chromatography. Isoelectric focusing revealed a pI of approximately 4.8 for both *M. avium* and *M. tuberculosis* HGPRT.

L. donovani XPRT was found to have K_m and k_{cat} values of 9.5 μM, 37 μM,

and 2.5 s^{-1}, 0.026 s^{-1} for xanthine and hypoxanthine respectively. Consequently, XPRT recognizes xanthine, hypoxanthine and guanine (data not shown) but only catalyzes the phosphoribosylation of xanthine efficiently.

K_m and k_{cat} values for *M. tuberculosis* HGPRT are 9.8 μM, 21.7 μM and 1.43 s^{-1}, 1.1 s^{-1} for hypoxanthine and guanine. No xanthine activity could be detected. Reported K_m values of human HGPRT are 7.6 μM and 4.3 μM for hypoxanthine and guanine.

The effect of pH on the phosphoribosylation of hypoxanthine by *M. tuberculosis* HGPRT was determined. Initial reaction rates were shown to be very slow below pH 6.0 and increasingly more rapid up to pH 9.0 with the enzyme active over a wide range of pH. Changes in magnesium concentration had a more pronounced effect on the HGPRT conversion of hypoxanthine to inosine monophosphate. Low concentrations of 0.5 to 2.0 mM allowed minuscule rates. A range of 5 - 40 mM $MgCl_2$ showed a characteristic bell-shaped curve with peak activity at 20 mM. At greater concentrations the magnesium divalent cation became inhibitory and resulted in a linear decline in enzyme activity. Substitution of cobalt or manganese augmented HGPRT activity above that seen with magnesium at 5 mM while other cations including calcium, copper, and iron were not effective alternatives. Zinc allowed phosphoribosylation *albeit* in a much reduced capacity.

Cultures of *M. avium* and *M. tuberculosis* were screened against twenty-nine purine analogs donated by Burroughs Wellcome. No drug was effective against *M. avium* however 6-mercaptopurine, 6-thiocyanopurine, 2-amino 6-thiopurine, Imuran, and 6-iodopurine inhibited growth while 2-amino 6-isothioaminopurine, 2-amino 6-iodopurine, and 6-nitrosomethylpurine killed *M. tuberculosis*.

INTRODUCTION

A. Structure-based drug design: The method traditionally used for drug discovery is pragmatic selection of either naturally occurring or chemically synthesized compounds against an individual infectious agent based on some reasonable hypothesis. For example, Gertrude Elion, George Hitchings and James Black shared a Nobel Prize for their work on the synthesis and testing of thousands of purine compounds beginning at a time when the structure of DNA was not yet understood. Even basic DNA metabolism had not been elucidated. For them, it was a "logical" step to attempt to produce "anti-metabolites" of purines as they had been shown to be a component of DNA. Their hope was to stop rapidly growing cells such as cancer. These heroic scientists did not wait until all of the biochemical pathways were determined. What Hitchings called "Enlightened Empiricism" was basic knowledge and serendipity as important integral parts of scientific discovery (Elion, 1985). Empirical derivation of therapeutically relevant agents has certainly been successful, yet the result is a paucity of knowledge regarding the underlying molecular mechanisms of drug action. Consequently, any effort to counter detrimental side-effects or to increase treatment effectiveness must be taken on rather blindly.

The emergence of multiple drug resistance has exacerbated the need for more efficacious and selective chemotherapeutic agents. As basic biochemical

and metabolic pathways continue to be characterized in infectious organisms, there has been an increased focus on specific targets which exhibit unique differences between parasite and host. Attempts to produce compounds with selective activity could possibly eliminate the need to screen hundreds of thousands of compounds that justify no *a priori* reason to test. Discovery of novel inhibitors to *Lactobacillus casei* thymidylate synthetase (Shoichet et al., 1993), *Schistosoma mansoni* cercarial elastase (Cohen et al., 1991), *Plasmodium falciparum* trophozoite cysteine protease (Ring et al., 1993), influenza virus sialidase (von Itzstein et al., 1993) and HIV protease (for reviews see: Wlodawer and Erickson, 1993; Vacca, 1994; Kempf, 1994; Carr and Cooper, 1996; Kaplan, 1996) represents successful attempts at structure-based drug design. Many more are in the early stages of discovery and development.

As methods to analyze the physical parameters of proteins and molecules are advancing, barriers to structure-based drug design will be overcome. Production of large amounts of purified recombinant protein is now routine laboratory practice and has facilitated the acquisition of structural information from circular dichroism, nuclear magnetic resonance and x-ray crystallography techniques. In conjunction with sophisticated computer programs such as DOCK 3.0, the structures can be used to screen chemical databases for potential inhibitors and alternative substrates (Shoichet et al., 1992). Although absolute

atomic assignments in a tertiary structure is optimal, molecular modeling based on the structure of similar molecules can also help predict an energy minimized topology. Once lead compounds are identified, testing can be easily initiated in both *in vitro* and *in vivo* systems prior to carrying out trials of these agents in animal models.

1. Approaches to drug design: Large pharmaceutical corporations in democratic countries with a plethora of funding to finance probable monetarily rewarding chemotherapuetica tend to employ any means necessary to increase their likelihood of prosperity. The result is that diseases of the affluent usually receive sufficient attention and there is often an abundance of available treatment. For diseases under-represented in the general population or mostly common to persons residing in developing countries, a total lack of, or outdated treatment may often be the only recourse. Unfortunately, many of the organisms responsible for largest percentages of annual worldwide morbidity and mortality can be classified in the latter category (Murray and Lopez, 1994). About three million human beings die from tuberculosis infections each year. Leishmaniasis claims an additional 55,000 annually. Neither infection can boast of reliable or generally available treatment and the development of such agents have not been a priority of pharmaceutical companies for at least 30 years. The emergence of drug resistance, and especially multiple resistance, with resulting therapeutic

failure raises devastating hindrances to successful therapy. Consequently, as the basic biology of a given infectious organism is studied, a continuously increasing number of approaches can be initiated against appropriate targets for drug design in an attempt to circumvent catastrophic possibilities of multiple drug resistances.

The most effective targets for chemotherapeutic agents are ones considered essential for the survival of the parasite but not the host. However, general differences in metabolic pathways, substrate specificity and inhibition profiles can ultimately be capitalized upon when attempting to generate an effective treatment for a given disease. Physical differences on a cellular level may exist between a host and parasite, such as those obvious between prokaryotes and eukayotes, and can make reasonable targets for drug design. Enzymes and their metabolites are often highly conserved throughout evolution yet many examples can be found to be missing either in a host or parasite. Sometimes pathways have unique subcellular locations or are arranged in a peculiar order, or isofunctional enzymes have evolved to ensure "survival of the fittest."

Other considerations necessary when "designing" a drug include ease of absorption, length of action, and specificity with minimal side effects. Since the research described in the present document focuses on the intracellular parasites, *Leishmania donovani, Mycobacterium avium* and *Mycobacterium tuberculosis*, further discussion of drug design approaches will be limited to their scope.

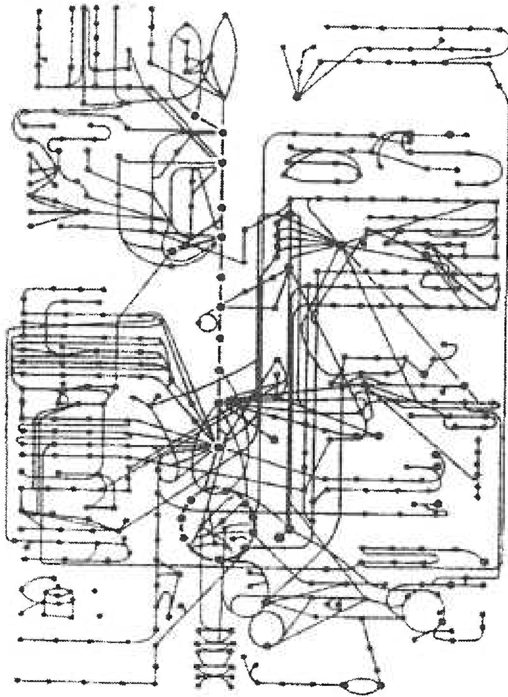
Leishmania donovani: Although the current status of antileishmanial therapy is gloomy, due to limited available drugs for treatment, their degree of toxicity, poor absorption and duration of action, the future holds promising possibilities. Fairlamb (1989), in an eloquent review, described novel biochemical pathways of parasitic protozoa, including *Leishmania donovani*. Figure 1 shows a line diagram of pathway similarities and differences between *Trypanasoma brucei* (a member of the family Trypanosoma to which *L. donovani* belong) and humans. As is easily discernable, there are many unique pathway differences which could be capitalized upon for a structure-based research protocol. Not indicated are distinctive non-metabolic molecules and atypical locations of some pathways.

Many initial investigations of basic biological features have uncovered targets for chemotherapeutic investigation. Glycosomes are unique organelles found in all members of the order Kinetoplastida (Opperdoes and Borst, 1977). Morphologically, they resemble other microbodies such as mammalian peroxisomes and plant glyoxysomes but differ in content and capacity. They are primarily glycolytic powerhouses and are involved in β-oxidation of fatty acids and biosynthesis of ether lipids, and also serve to compartmentalize the pyrimidine biosynthetic enzymes, orotate phosphoribosyltransferase (OPRTase) and orotidine-5'-decarboxylase (ODCase), and some purine salvage enzymes

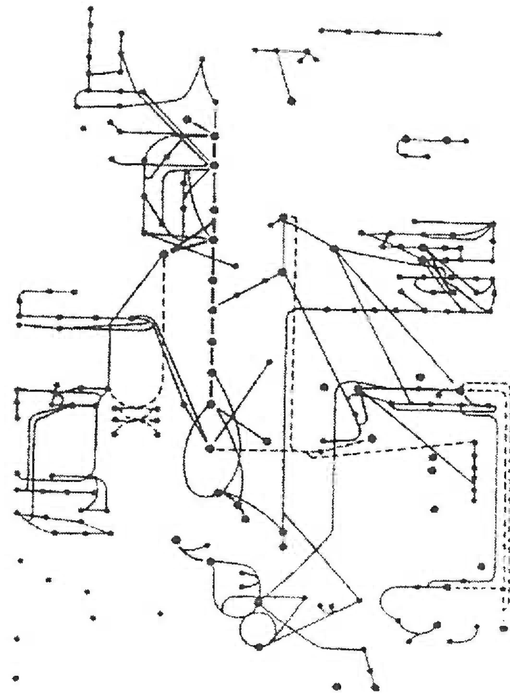
Figure 1: Comparison of major metabolic pathways from human (A) and *Trypanosoma brucei* (B). (Fairlamb, 1989)

Figure 1A is a line diagram depicting mammalian metabolism. The central importance of the glycolytic and tri-carboxylic acid pathways are emphasized with heavy lines. Each dot represents a single metabolite with interconnecting lines representing enzyme catalyzed reactions. Novel pathways in the bloodstream form of *T. brucei* (Figure 1B) are shown by dotted lines.

A. Human



B. ***Trypanosoma brucei***



(Fairlamb 1989); (Hwang et al., 1997), all of which may be excellent therapeutic targets. Unlike most other eukaryotic peroxisomes, *Leishmania sp.* glycosomes do not contain catalase or peroxidase (Opperdoes and Michels, 1993). The resident proteins are translocated through the single bilayer membrane guided by a novel internal sequence or a tripeptide C-terminus known for the ability to signal the glycosome. The requirements of the latter are reasonably relaxed. The first amino acid can be any small, neutral amino acid; the second should be capable of forming hydrogen bonds; and the third must be hydrophobic (Sommer and Wang, 1994). The assumed advantage of compartmentalization is the increased concentration of enzymes and their intermediates (Wang, 1995).

Inhibition of glycolysis is deleterious to *T. brucei* survival and selective targeting of specific enzymes may lead to new anti-protozoan therapeutica. The pathway itself has some novel features including the fact that dihydroxyacetone phosphate can be converted to methylglyoxal, S-D-lactoglutathione, and eventually to the D-isomer of lactate through the sequential actions of methylglyoxal synthase and glyoxalase I and II (Darling and Blum, 1988). Additionally, disruption of some, but not all, enzyme targeting to the glycosome could abolish the glycolytic pathway as phosphorylated intermediates trapped inside might not be processed further.

Another organelle unique to the Order Kinetoplastida, and for which they

begot their name, is the kinetoplast. Located proximal to the flagellar pocket, it contains episomal DNA necessary for production of proteins resident in its single long, slender mitochondria. Approximately 10,000 minicircles contain information for guide RNA's to correctly edit the genes encoded by the 20-50 maxicircles (Feagin et al., 1988). RNA editing is complex. Significant contributions to the understanding of editing mechanisms have been made (Feagin et al.,1988; Kable et al.,1996; Seiwert and Stuart, 1994). Even as the machinery involved is being experimentally unravelled, it is an obvious target of chemotherapists.

Mature mRNAs of *L. donovani* are produced in a fascinating manner prior to translation (Agabian, 1990). Polycistronic pre-mRNA's are transpliced with a capped, 39 nucleotide miniexon splice leader to an AG acceptor site adjacent to a pyrimidine-rich upstream region. Poly(A) addition is specified by the position of the next downstream splice site (LeBowitz et al., 1993). As the first step in gene regulation, these splicing events present us selective targets for drug development.

Translation initiation ensues after a ribosomal complex forms around the message. Paromonomycin, an amino glycoside, has been shown to inhibit the dissociation of both cytoplasmic and mitochondrial ribosomes into their respective 67S, 49S and 60S, 39S complexes thereby causing mistranslation

(Maarouf et al., 1995). The drug is currently in clinical trials. Interestingly, neomycin, a close structural analog, also disrupts translation *in vitro* yet is not active against visceral leishmaniasis.

Two prominent *L. donovani* surface molecules that have been reasonably well characterized are gp63, a surface protease, and lipophosphoglygan (LPG), a linear chain of galactose-mannose-phosphate repeats tethered to the cell membrane by a glycosylphosphatidalinositol anchor. Immunoprotection of mice against cutaneous leishmaniasis by innoculation with a BCG producing recombinant gp63 has been demonstrated (Connell et al., 1993) while LPG has been shown to be essential for survival in macrophage phagolysosomes (Brandonisio et al., 1994). The LPG biosynthetic enzymes could eventually be "rational" targets and gp63 may have vaccine potential.

Anabolism of some unusual 24-alkyl sterols such as ergosterol in *L. donovani* represents the identified target of amphotericin antiparasitic action (Chance, 1995). Unfortunately, unpleasant side-effects including anemia occur due to actions on human cholesterol as well. Kidney failure is common. A more specific drug is needed.

Polyamine metabolism has been considered an established target for parasitic protozoan chemotherapy since the 1970s (Bacchi and Yarlet, 1995). Polyamines are low molecular weight, positively charged, molecules that are

essential for cell growth, cell division and differentiation due, in part, to their ability to stabilize tRNA and DNA. Mammalian and *L. donovani* polyamine metabolism differ in many significant ways: 1. The half live of the enzyme initiating polyamine anabolism, ornithine decarboxylase (ODC), is very short in mammals and considerably longer in typanosomatids. 2. ODC converts ornithine to putrescine, from which spermidine and spermine are produced. The mammalian pathway is not feedback regulated and the polyamines can be interconverted whereas leishmanial growth is inhibited (i.e. regulated) by bis(benzyl)polyamines (Bauman et al., 1990) and no back conversion has been shown. 3. In kinetoplasts but not in mammalian cells, spermine conjugates with glutathione to form a novel reducing molecule, trypanothione (N^1,N^8-bis(glutathionyl) spermidine (Fairlamb, 1985). Trypanothione non-enzymatically reduces oxidized glutathione and is then enzymatically reduced by a specific trypanothione reductase (TR). TR is 41% identical to mammalian glutathione reductase yet exhibits absolute specificity for trypanothione and does not recognize glutathione as a substrate (Fairlamb, 1985).

DFMO, DL-α-difluoromethylornithine, is a suicide inhibitor of ODC and shows a low *in vitro* EC_{50} of 30 μM (Coons et al., 1990) yet is not an efficacious treatment for leishmaniasis. *L. donovani* have effective polyamine transporters, unlike other related *Trypanosomatidae* on which DMFO is active, and may be

able to overcome the block by utilizing host polyamines (Bacchi and McCann, 1987). Consequently, development of a polyamine pathway subversive substrate is a more attractive possibility than production of a general inhibitor. ODC, TR or SAMDC (S-adenosylmethionine decarboxylase), which provides substrate for spermidine synthesis, are both likely target enzymes.

Folates, 1-carbon donors in the synthesis of choline, serine, glycine, methionine, purines and dTMP, are produced *de novo* in most protozoans while for humans they represent a dietary requirement making folate metabolism another interesting chemotherapeutic target in leishmania (Marr and Ullman, 1995). Two classes of inhibitors, although not yet tested in *L. donovani,* have been effective in elimination of other parasitemias, including malaria, toxoplasmosis and pneumocystosis (Katz et al., 1989). The first consists of the diaminopyrimidines, inhibitors of the folate synthetic enzyme, dihydropteroate synthase. Due to their lack of a mammalian target, these inhibitors are virtually non-toxic. The second class, *p*-aminobenzoic acid antagonists, capitalizes upon the enzyme specificity of the single bifunctional enzyme dihydrofolate reductase - thymidylate synthase (DHFR-TS). Neither of the two distinct enzymes in humans are affected by this second class of drugs. *Leishmania*, like other protozoans, have recently been shown to synthesize folate (Beck and Ullman, 1990, 1991) implying that these pathways might also serve as therapeutic targets.

Recently, an established antitumor drug, camptothecin, known to act upon eukaryotic topoisomerase I, was tested against *L. donovani*. An *in vitro* EC_{50} value of 3.2 μM was determined (Bodley and Shapiro, 1995). Camptothecin may represent a new lead compound for chemotherapy and\or an important reagent to study topoisomerase I activity and study of this compound may provide a lesson against established dogma (i.e. targets for chemotherapeutica **do not** necessarily have to be unique, sometimes they simply need to interfere with the survival of the offending organism). Certainly human topoisomerase I is effected by camptothecin therapy, and cells rapidly dividing cease to do so. The compromise allows elimination of tumors and parasitic infections and as a side-effect puts normal cell division on hold. The draw back, of course, is that a therapy such as this would be less appropriate for children than for adults.

Mycobacterium avium* and *Mycobacterium tuberculosis: Designing successful chemotherapeutica for prokaryotic mycobacteria might seem easier than for eukaryotic parasites. However, their capacity to lie dormant for years and their generally refractory nature toward drug permeation could pose serious limitations. If general differences between prokaryotes and eukaryotes are targeted, symbiotic bacteria are also eliminated. For short durations, it may be inconsequential as is common for many general antibiotics, but to ensure cure from quiescent mycobacteria, extensive treatment is obligatory and the side-

effects are significant. Currently, the biggest hindrance to drug design is the obvious dearth of understanding the basic biology of mycobacterial organisms. Recent recrudescence of TB has stimulated primary research and future possibilities appear more promising with the limitation that protocols involving *M. tuberculosis* bare real health risks and generally are far from facile.

On a relatively crude level, two intriguing aspects of mycobacteria have been targeted as possible sites for drug design:

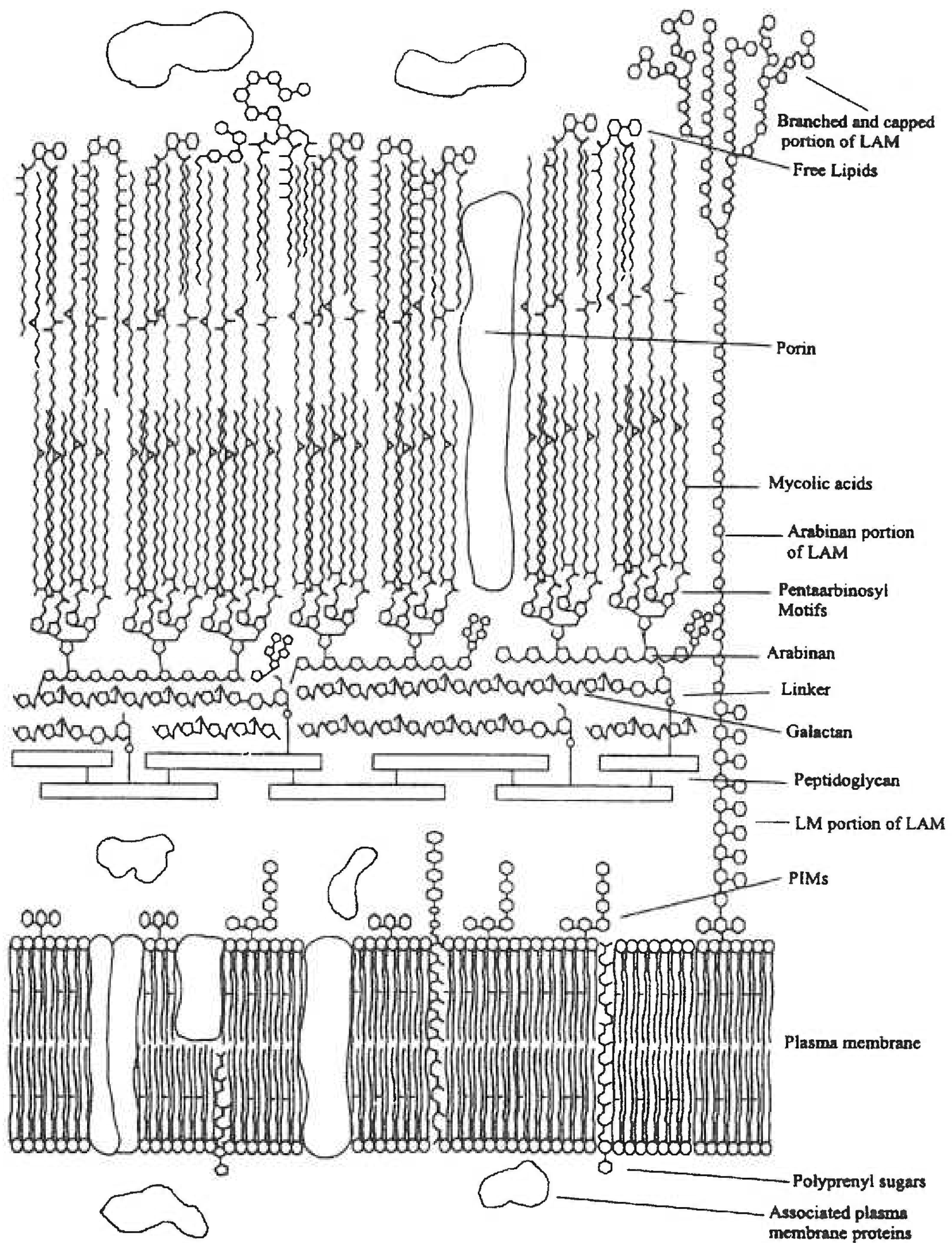
1. The complex cell wall that controls their hydrophobicity, relative diminutive size, acid fast staining, and resistance to chemical injury by alkali, acids and other disinfectants that are typically bacterialcidal, sets mycobacteria apart from all other known bacteria. The plasma membrane also contains a novel fatty acid known as tuberculostearate (10-methyl stearic acid). A model of the putative cell envelope is shown in Figure 2. *Mycobacterium avium* is more refractory to penetration than *M. tuberculosis* due to it's ability to synthesize serovar-specific glycopeptidolipid (GPL) antigens (Rastogi et al., 1996).

Most of the molecules have been isolated and identified yet the metabolic machinery for production of such intricate armor remains to be determined. Elucidation and characterization of the biosynthetic pathways of these molecules could potentially provide extremely specific targets for drug design.

2. Secretory proteins have been postulated to help mycobacteria adapt to

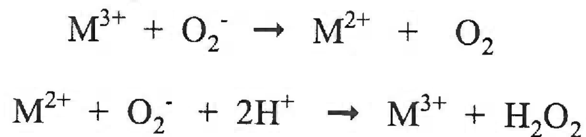
Figure 2. Mycobacterial cell envelope.

M. tuberculosis cell envelope



their hostile environment. Anderson and co-workers (1991) studied protein accumulation in short term cultures. Six antigens predominated after three days while others accumulated more slowly. Proteins released into the media were categorized into three groups; excreted proteins that rapidly appeared but were scarce in intact bacilli; secreted proteins of the outer cell wall that were gradually released into the media and accumulated over time; and cytoplasmic proteins released in late log phage as dead bacteria lysed.

One of the excreted proteins identified was superoxide dismutase (SOD). This enzyme is capable of catalyzing a two step reduction of oxygen free radical (Lavelle et al., 1977; McAdam et al., 1977).



$$M^{3+} + O_2^- \rightarrow M^{2+} + O_2$$

$$M^{2+} + O_2^- + 2H^+ \rightarrow M^{3+} + H_2O_2$$

Analysis of the X-ray structure of the iron dependent SOD from *M. tuberculosis* revealed novel quaternary structure differences not seen in previously solved structures from five other organisms including the human enzyme (Cooper et al., 1995). Unlike the N-terminal αα-turn responsible for the tetrameric association of two typical SOD dimers, *M. tuberculosis* SOD dimerization occurs through an internal loop region that connects to two outer β-strands to form a three membered β-sheet. Chemotherapeutic dimunition of excreted SOD may hamper the ability of *M. tuberculosis* to survive the host immune generation of

antimicrobial reactive oxygen intermediates.

Another secreted component of the short-term culture was partially characterized and found to be PhosS, a phosphate-binding protein. In gram-negative bacteria such as *E. coli* it is normally found in the periplasmic space but since mycobacteria lack a partition between the cell wall and membrane it is likely that PhosS is secreted through the membrane and loosely resides in the cell wall where it is efficiently located to effect phosphate metabolism (Andersen et al., 1991).

Glutamate synthetase may represent another solid target for drug therapy. Its action is two-fold. As an extracellular protein it influences the ammonia level, and consequently the pH, in infected host cells. Intracellular synthesis of glutamine is utilized for the production of poly(L-glutamic acid/glutamine) found in pathogenic but not non-pathogenic mycobacterial cell walls (Harth, 1994).

Iron is necessary for life in all known organisms with the exception of lactobacilli. Siderophores, iron chelators, are excreted by mycobacteria to facilitate iron acquisition. Exochelin, a siderophore, is known to "steal" iron from host cell transferrin or ferritin and donate it to the hydrophobic lipoprotein mycobactin T for transport across the cell wall (Wheeler and Ratledge, 1994). Iron is then incorporated into cytochromes and other heme-containing proteins as

well as enzymes that utilize it in their catalytic site such as aconitase, ribonucleoside reductase, and additional hydroxylases and oxidases. Whether one can specifically target iron acquisition in mycobacteria is an interesting question. Specific chelation is probably not feasible as host iron would also be affected yet blocking specific uptake of iron through the cell envelope may be possible and remains an active area of research (Wheeler and Ratledge, 1994). As transcriptional regulation of the iron-siderophores becomes more well understood, pharmacological targeting at this level might also prove successful.

Interestingly, targeting host cell ornithine metabolism may be a reasonable approach. Almost a half a century ago Hirsch and Dubos discovered the tuberculostatic effects of the polyamine, spermidine, (Hirsch and Dubos, 1952) and showed that an enzyme was responsible for its killing effects (Hirsch, 1953). Amino aldehyde, ammonia and hydrogen peroxide are now known to be the oxidative deamination products of catalysis by polyamine oxidase (Morgan 1987). Although oxidation of polyamines is also cytotoxic to mammalian cells, host cell death is not necessary for it's mycobacterialcidal effects. The polyamines, spermine and spermidine, play an important role in the initiation and regulation of cell proliferation and differentiation (Heby, 1986), activation of macrophages (Messina et al., 1992) and apoptosis (Chayen et al., 1990). ODC, the rate limiting enzyme in polyamine synthesis, is increased in activity (and accumulation of

mRNA) by treatment of monocytes with IFN-γ (Messina, 1990). The selective targeting of mycobacterial host cells may represent an exciting alternative avenue for therapy.

As the basic biology of the mycobacteria become understood, a greater capacity to effect therapy will be concurrent. Mycobacterial research can be a formidable task hindered by technological limitations and health risks to laboratory personnel. As a result, even fundamental metabolic pathways and porin transporters have not been well studied. Most have been inferred by the ability of the bacteria to grow in selected medias. Limited data is available on mycobacterial purine biosynthesis and salvage (Wheeler,1987, 1990). These studies did not include *M. tuberculosis. Mycobacterium leprae,* the causative agent of leprosy, was found to be auxotrophic for purines while other mycobacteria including *M. avium* and *M. microti* could salvage purines or synthesize them *de novo*. These findings are not suprizing since *M. leprae* fails to grow in defined culture mediums while *M. avium* and *M. microti* grow with the addition of simple carbon sources. *M. tuberculosis* can also grow in minimal media. Since *M. avium* and *M. tuberculosis* are not obligate intracellular pathogens, they have seemingly kept all metabolic machinery intact.

What is very clear from these studies is that apparent regulation of purine biosynthesis and salvage is lacking. Little change in biosynthetic indices (rate of

synthesis to salvage), regardless of growth conditions, seems to indicate an important role for both pathways. Determination of the substrates available for parasite acquisition reveals that most metabolic substrates are readily available with the exception of adenine which is available at very low levels (Barclay and Wheeler, 1989). Given the high G + C content of mycobacterial and leishmanial DNA, it is intriguing to contemplate that these organisms may have evolved to enable themselves to utilize the much needed, but scarce, adenine for energy supplies in lieu of DNA production.

2. Purine Salvage Pathway as a Therapeutic Target: Five years before he shared the 1959 Nobel Prize for his work on a more "sexy" enzyme, DNA polymerase, Arthur Kornberg published together with Irving Leiberman and Ernest Simms four incredible back-to-back papers in the Journal of Biological Chemistry describing the enzymatic synthesis and properties of 5-phosphoribosyl-pyrophosphate, and the purine and pyrimidine nucleotides, including their mono, di and tri phosphorylated forms (Kornberg et al., 1955a, 1955b; Lieberman et al., 1955a, 1955b). The purine phosphoribosyltransferases described became known as part of the salvage pathway. The name "salvage" implied a relatively unimportant role, sort of a back up process to shuffle energy resources around.

Then Micheal Lesch and William Nyhan discovered a familial syndrome of uric acid metabolism and central nervous system disfunction characterized by

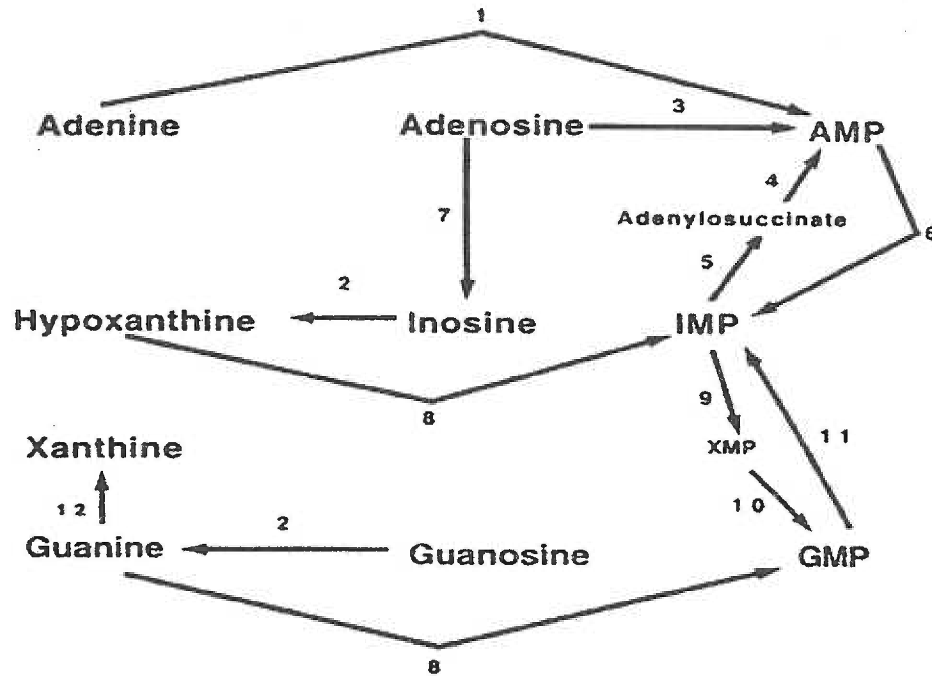
spasticity, choreoathetosis, mental retardation, and compulsive self-mutilation of fingers and lips (Lesch and Nyhan, 1964). Four years later a mutation in the enzyme hypoxanthine-guanine phosphoribosyltransferase was implicated in Lesch-Nyhan disease (Seegmiller et al., 1967). The view of the importance of the salvage pathway began to change. As the first metabolic pathway associated with a mental disorder, purine salvage served to alter the medical outlook on behavioral aberrations. The complete chemical basis for the neurological consequences of the reduction or absence of hypoxanthine-guanine phosphoribosyltransferase activity to this date remain evasive, yet it's mystery reminds scientists that the full significance of the salvage pathway has still not been elucidated.

Purine nucleotides are key components in both DNA and RNA, the coenzymes NAD^+, FAD and CoA, and they serve as the universal cellular energy source while also functioning as metabolic regulators. Understanding the magnitude of their impact on life in general, it makes sense that organisms would evolve a pathway to "salvage" these molecules to avoid wasting valuable energy. Although salvage pathways from diverse organisms play essentially the same role, fundamental differences are apparent. Figure 3 represents a comparison between the human and *L. donovani* salvage pathways. The phosphoribosylation reaction of biologically relevant purines (Fig. 4) is shown in Fig. 5. The pathway in mycobacteria has not yet been defined.

Figure 3: Purine Salvage Pathways (Berens et al., 1995).

Enzymes are as follows: 1) adenine phosphoribosyltransferase (APRT) 2) nucleoside phosphorylase 3) purine nucleoside kinase 4) adenylosuccinate lyase 5) adenylosuccinate synthetase 6) AMP deaminase 7) adenosine deaminase 8) hypoxanthine-guanine phosphotransferase (HGPRT) 9) inosine monophosphate dehydrogenase (IMPDH) 10) GMP synthase 11) GMP reductase 12) guanine deaminase 17) xanthine phosphoribosyltransferase (XPRT) 18) adenine deaminase 19) purine nucleoside hydrolase.

A. Human Salvage Pathway



B. *Leishmania donovani* Salvage Pathway

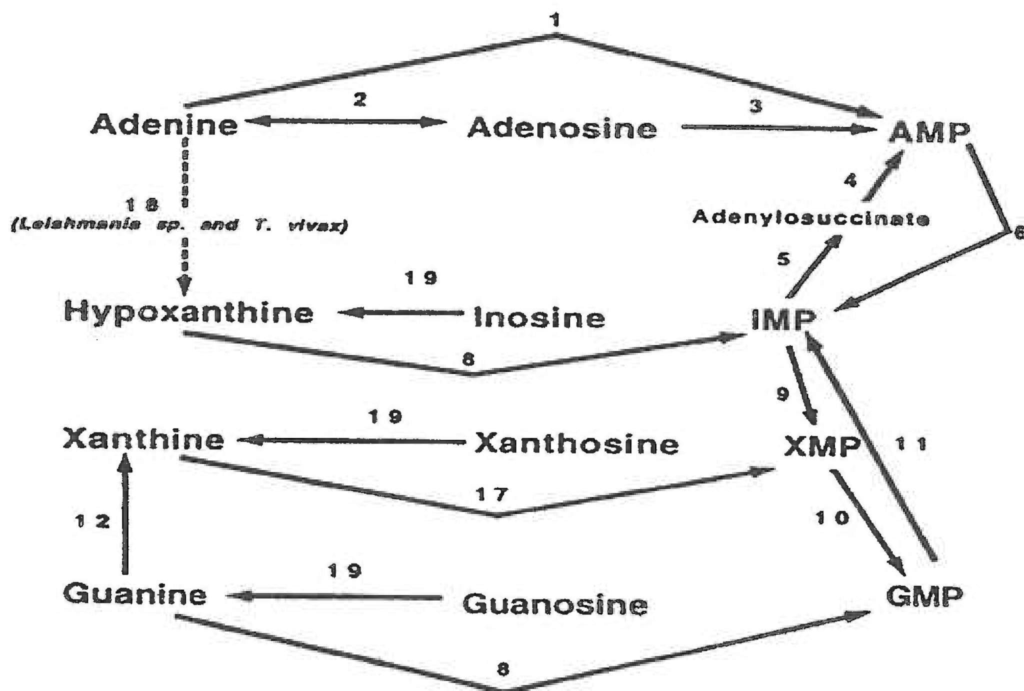
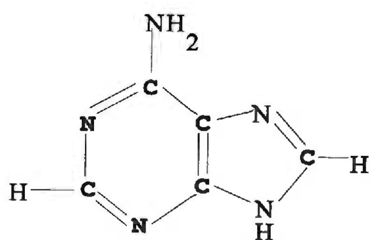
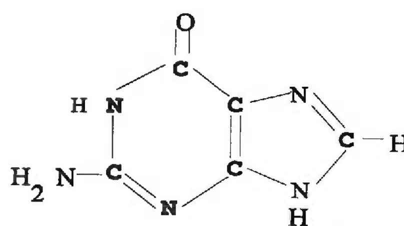


Figure 4: Purines

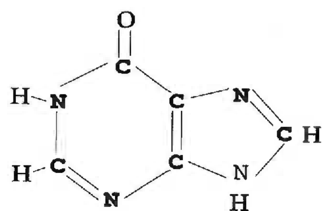
Purines



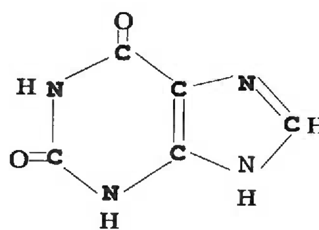
Adenine



Guanine

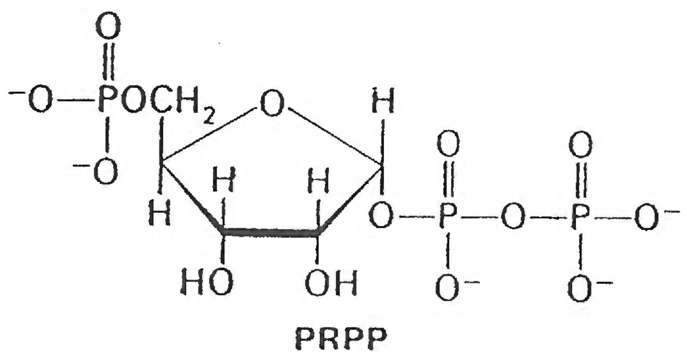


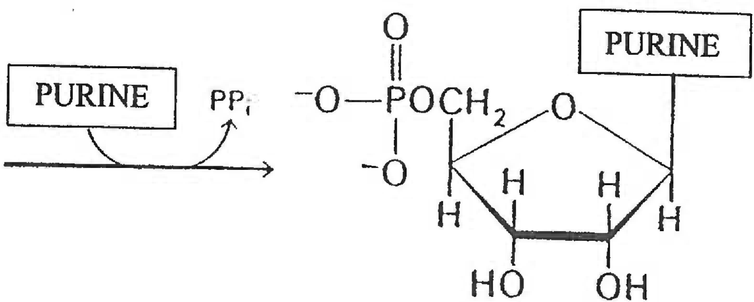
Hypoxanthine



Xanthine

Figure 5: Phosphoribosylation Reaction:


PRPP



Purine ribonucleotide

The salvage pathway has long been considered an attractive target for chemotherapy due primarily to the fact that all parasitic protozoans studied to date are auxotrophic for purines. The assumption was that the inhibition of a key enzyme would disrupt the pathway and lead to the demise of the organism. In spite of this belief, essential nutritional roles for each salvage enzyme have never been established. Recently it was shown that *Toxoplasma gondii* and *L. donovani* mutants deficient in HGXPRT and HGPRT respectively are perfectly viable (Roos et al, 1995, Hwang and Ullman, 1997). Regardless, many other arguments can be made that the salvage pathway is an appealing target for structure-based drug design and are described as follows: Intracellular pathogens including *L. donovani, M. avium* and *M. tuberculosis* pose unique considerations for design of chemotherapeutica. Two important and obvious ones are their specific intracellular location (i.e., a phagosome within a macrophage) and their novel external barriers to permeation. Drugs must have the capacity to penetrate both restrictions and yet remain satisfactorily concentrated as well as active to be efficacious. Purine analogs are likely to be structurally related enough to sufficiently exploit the natural transporters of the biologically relevant purines. Once access has been gained, phosphoribosylation by a PRTase could essentially trap a "pro-drug" and subsequent cytotoxic effects should be limited to the parasite. For this to occur to the parasite and not the host, either substrate

specificity or pathway differences need to exist. Given the diversity of the salvage pathways and PRTases in particular (Fig. 6) studied to date, it is a reasonable assumption (Berens et al., 1995, Ullman and Carter, 1995).

Evidence to support this hypothesis is that metabolism of several pyrazolo-pyrimidine analogs of natural purines (Fig. 7) to the nucleotide level by *L. donovani* has been demonstrated not to occur in humans. Of these, allopurinol is considered the best "lead" compound (Marr, 1991) and it's chemotherapeutic specificity is three-fold: 1) *L. donovani* HGPRT catalyses the phosphoribosylation of allopurinol as if it were a purine while the human enzyme does not. Conversion to oxipurinol and allopurinol-1-riboside in humans result in excretion (Fig. 8); 2) Inhibition of both succinyl AMP- synthase and GMP reductase completely blocks interconversion of nuceotide pools (Marr, 1991); 3) In *L. donovani* parasites, further processing occurs with eventual nucleotide incorporation into RNA (Marr, 1991). A concomitant breakdown of the aberrant mRNA results in an increase of nucleotides in the cytoplasmic pool, accompanied by inhibition of protein synthesis.

Consequently, enzyme specificity, pathway differences, and inhibition profiles all play a role in the efficacy of allopurinol against *L. donovani* which makes the purine salvage pathway an especially exciting area for further protozoan research as well as other organisms, such as the mycobacteria.

Figure 6: Comparison of HGPRT's (Ullman and Carter, 1995).

6a. Alignments of the primary structures of *Toxoplasma gondii, Plasmodium falciparum,* human, *Crithidia fasciculata, Leishmania donovani, Trypanosoma brucei, Trypanosoma cruzi* and *Tritrichomonas foetus* HGPRT. Note the low degree of identity even within regions contributing to the active site. Box I: Found to surround the ribose-phosphate moiety of the product. Box II: PRPP binding motif. Box III: Associates with purine ring.

6b. Identity comparison of the HGPRT amino acid sequences.

A. Primary structure alignment

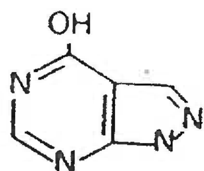
```
                                                                                                                 I.
T. gondii       MASKPIEDYGKGKGRIEPMYIPDNTFYNADDFLVPPHCKPYIDKILLPGGLVKDRVEKLAYDIHRTYFGEE......LHI | ICILKGSRGFFNLL | IDYLAT  94
P. falciparum   ..MPIPNNPGAGENAFDPVFVKDDDGYDLDSFMIPAHYKKYLTKVLVPNGVIKNRIEKLAYDIKKVYNNEE......FHI | LCLLKGSRGFFTAL | LKHLSR  92
Human           ..........MATRSPGVVISDDEPGYDLDLFCIPNHYAEDLERVFIPHGLIMDRTERLARDVMKEMGGHH.......IVA | LCVLKGGYKFFADL | LDYIKA  84
C. fasciculata  ..................MSNAA...SPATSAAPVRHYPMSCRTLATQEQIWSATAKCAKQIAEDYKQYNLSD.ENPLYL | LCVLKGSFMFTADL | ARFL..  76
L. donovani     .................MSNSAKSPSGPVGDEGRRNYPMSAHTLVTQEQVWAATAKCAKKIAEDYRSFKLTT.DNPLYL | LCVLKGSFIFTADL | ARFL..  79
T. brucei       ...................................MEPACKYDFATSVLFTEAELHTRMRGVAQRIADDYSNCNLKPLENPLVI | VSVLKGSFVFTADM | VRIL..  67
T. cruzi        .....................................MPREYEFAEKILFTEEEIRTRIMEVAKRIADDYKGKGLRPYVNPLVL | ISVLKGSFMPTADL | CRAL..  65
T. foetus       ...................................MTETPMMDDLERVLYNQDDIQKRIRELAAELTEFYED......KNP.VM | ICVLTGAVFFYTDL | LKHL..  61
                                                              *  .        *     **     |   *.*.   *     |

                                                                    II.
T. gondii       IQKYSGRESSVPPFFEHYVRLKSYQNDNSTGQLTVLS.DDLSIFRD | KHVLIVEDIVDTGFTL | TEFGERLKAVGPKSMRIALVEKRTDRS.NSLKGDFVG  192
P. falciparum   IHNYSAVEMSKPLFGEHYVRVKSYCNDQSTGTLEIVS.EDLSCLKG | KHVLIVEDIIDTGKTL | VKFCEYLKKFEIKTVAIALFIKRTPLW.NGFKADFVG  190
Human           LNRNSDRSIPMTV...DFIRLKSYCNDQSTGDIKVIGGDDLSTLTG | KNVLIVEDIIDTGKTM | QTLLSLVRQYNPKMVKVALLVKRTPRS.VGYKPDFVG  180
C. fasciculata  ....CDEGVPVRI...EFICASSYGTDVKTSGEVRLLLDVRDPVEN | RHLLIVEDIVDSAITL | EYLKRFLNAKNPASLKTVLLDKPSGRK.VTLSVDYPV  168
L. donovani     ....ADEGVPVKV...EFICASSYGTGVETSGQVRMLLDVRDSVEN | RHILIVEDIVDSAITL | QYLMRFMLAKKPASLKTVLLDKPSGRK.VEVLVDYPV  171
T. brucei       ....GDFGVPTRV...EFLRASSYGHDTKSCGRVDVKADGLCDIRG | KHVLVLEDILDTALTL | REVVDSLKKSEPASIKTLAIDKPGGRK.IPFTAEYVV  159
T. cruzi        ....SDFNVPVRM...EFICVSSYGEGVTSSGQVRMLLDTRHSIEG | HHVLIVEDIVDTALTL | NYLYHMYFTRRPASLKTVLLDKREGRR.VPFSADYVV  157
T. foetus       ......DFQLEP...DYIICSSY.SGTKSTGNLTISKDLKTNIEG | RHVLVVEDIIDTGLTM | YQLLNNLQMRKPASLKVCLCDKDIGKKAYDVPIDYCG  149
                      ...    **      .     .   .   .      |  ..*..***.*.. *  |  .            ..  .  *          ..

                     III.
T. gondii       FS | IEDVWIVGCCYDFNEMFRDF | DHVAVLSDAARKKFEK.............................  230
P. falciparum   FS | IPDHFVVGYSLDYNEIFRDL | DHCCLVNDEGKKKYKATSL..........................  231
Human           FE | IPDKFVVGYALDYNEYFRDL | NHVCVISETGKAKYKA.............................  218
C. fasciculata  IT | IPHAFVIGYGMDFAEAYREL | RDVCVLKKEYYEKPA.........................SKL  208
L. donovani     IT | IPHAFVIGYGMDYAESYREL | RDICVLKKEYYEKPE.........................SKV  211
T. brucei       AD | VPNVFVVGYGLDYDQSYREV | RDVVILKPSVYETWGKELERRKAA.......GEAKR......  210
T. cruzi        AN | IPNAFVIGYGLDYDDTYREL | RDIVVLRPEVYAEREAARQKKQRAIGSADTDRDAKREFHSKY  221
T. foetus       FV | VENRYIIGYGFDFHNKYRNL | PVIGILKESVYT.................................  183
                   | .  ...*  .*.   .*.. |   ..                                      **
```

B. HGPRT % identity

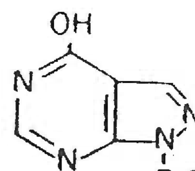
	Human	*Plasmodium falciparum*	*Trypanosoma brucei*	*Trypanosoma cruzi*	*Leishmania donovani*	*Crithidia fasciculata*	*Toxoplasma gondii*	*Tritrichomonas foetus*
Human	—							
P. falciparum	48	—						
T. brucei	28	31	—					
T. cruzi	35	32	52	—				
L. donovani	29	23	41	56	—			
C. fasciculata	30	24	43	56	78	—		
T. gondii	41	52	30	29	25	28	—	
T. foetus	32	29	33	37	33	34	26	—

Figure 7: The pyrazolopyrimidines.

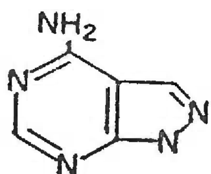
PYRAZOLOPYRIMIDINES



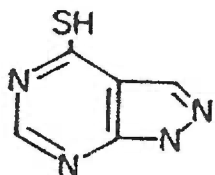
Allopurinol (HPP)



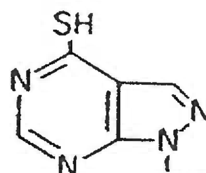
Allopurinol Riboside (HPPR)



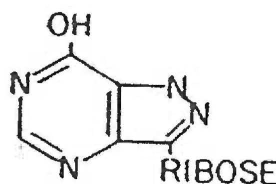
Aminopurinol (APP)



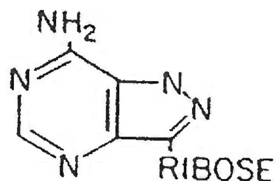
Thiopurinol (TPP)



Thiopurinol Riboside (TPPR)

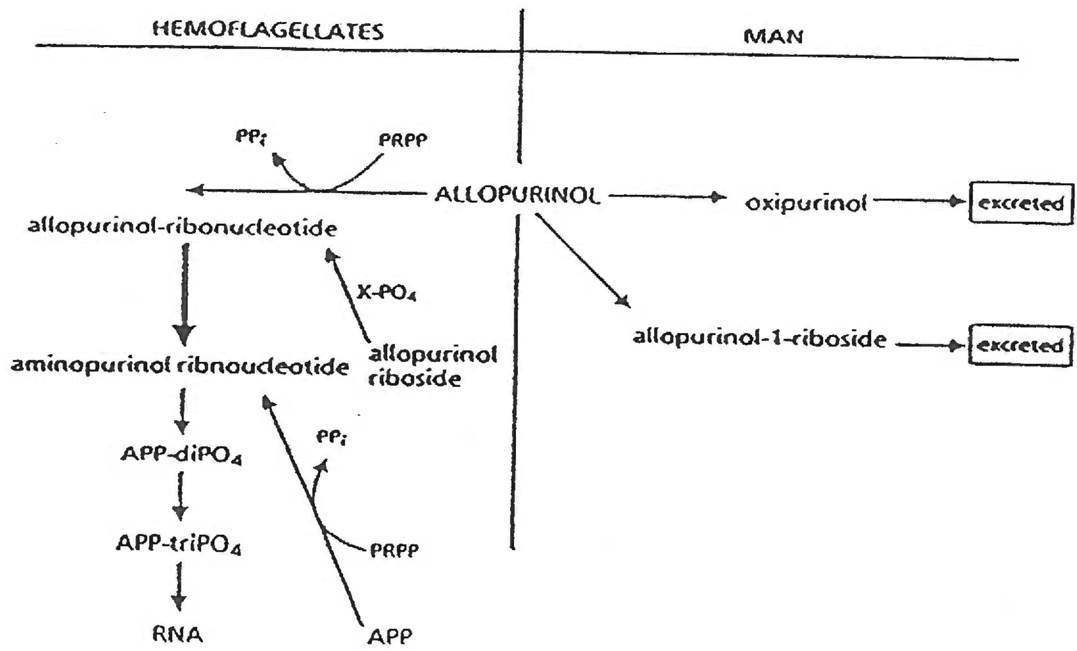


Formycin B



Formycin A

Figure 8: Comparative Allopurinol Metabolism (Marr, 1991).


HEMOFLAGELLATES
MAN
PPi
PRPP
ALLOPURINOL
oxipurinol
excreted
allopurinol-ribonucleotide
X-PO4
allopurinol-1-riboside
excreted
aminopurinol ribnoucleotide
allopurinol
riboside
APP-diPO4
PPi
APP-triPO4
PRPP
RNA
APP

B. ***Leishmania donovani*****:** *Leishmania donovani*, a digenetic flagellated protozoan, is the causative agent of visceral leishmaniasis or Kala-azar. Dipteran sand flies including *Lutzomia longipalpis* in the Americas and *Phlebotomus* species elsewhere (Molyneux and Ashford, 1983) transmit the parasite to humans and other vertebrates while taking a bloodmeal. The connection between Kala-azar and *Leishmania donovani* was discovered in 1903 when William B. Leishman, a Scotsman, noted indications of a disease that were distinct from pernicious malaria, a particularly common affliction, at the British military station of Dum-Dum near Calcutta. A low grade fever with hepatosplenomegaly and severe cachexia persisted despite antimalarial treatment (Leishman, 1903). Upon examination of splenic smears, distinct organisms were found which looked like the degenerating trypanosomes discovered in a dead rat. In Madras, C. Donovan from the Indian Medical Service read of Leishman's work and performed a spleen puncture on a patient presumably dying of chronic malaria and demonstrated that the parasites were identical in fresh tissue and therefore were not degenerate forms (Donovan, 1903). Sir Ronald Ross, later in 1903, officially named the genus and species *Leishmania donovani* in recognition of both doctors for their landmark discovery.

Nicolle (1908) discovered that other mammals, especially dogs, could serve as reservoirs for the parasite and the sandfly was officially incriminated as the

vector when Short, Smith and Swaminath (1931) successfully achieved transmission of the disease to hamsters. This was a monumental breakthrough considering the difficulty with sandfly survival through oviposition and refeeding under laboratory conditions. Other possible modes of transmission reported include transplacental, veneral and carnivory, but are considered somewhat controversial (Molyneux and Ashford, 1983). Amazingly, volunteers were utilized to prove that the *Phlebotomus* sandflies were indeed a vector for humans at a time when no effective drug was available for this potentially lethal pathogen (Swaminath et al., 1942).

Leishmania donovani is considered an Old World and New World parasite (see Figure 9) and it's worldwide distribution is shown in Figure10. A diverse range of clinical outcomes are possible from this single organism, including most commonly visceral leishmaniasis, infantile visceral leishmaniasis, post kala-azar dermal leishmaniasis, as well as mucocutaneous leishmaniasis (Africa), and, rarely, cutaneous leishmaniasis (Molyneux and Ashford, 1983).

1. Life cycle of *Leishmania donovani*: The infection of humans and other mammals begins with the bite of an infected sandfly (Figure 11). Slender metacyclic promastigotes migrate through the proboscis into the wound site and undergo receptor-mediated phagocytosis by macrophages. Some of the promas- tigotes may be lysed when the alternate pathway of the complement cascade is

Figure 9: Old and New World Species of *Leishmania* (Molyneux and Ashford, 1983).

The Old World *Leishmania* species parasitic in man.

Species	Main habitat	Main vertebrate host range	Main vectors	Disease spectrum
L. aethiopica	Tropical montane forest and gorges	Rock hyrax	*P. longipes*, *P. pedifer*	OS (chronic, dry) DCL
L. donovani	Temperate to tropical, mountains to plains, sylvatic to synanthropic, mostly sub-humid, warm summers, alluvial soil	Wild Canidae, savannah rodents (Africa), domestic dogs, man (India)	*P. (Larroussius)* spp., *P. argentipes*, *P. martini*?, *P. chinensis*	VL, MCL (Africa), CL, infantile VL, PKDL
L. major	Semi desert subtropics and tropics	Desert and savannah rodents: *Rhombomys*, *Psammomys*, *Arvicanthis*	*P. papatasi*, *P. (Paraphlebotomus)* spp.	OS (rural, wet)
L. tropica	Synanthropic in semi-arid subtropics	Man	*P. sergenti*	OS (urban, dry), Leishmaniasis recidivans, VL (rare)
L. sp. (Namibia)	Semi-desert, rocky areas	Hyrax?	*P. rossi*	OS (chronic)

DCL = Diffuse cutaneous leishmaniasis. Infantile VL = Infantile visceral leishmaniasis. MCL = Mucocutaneous leishmaniasis. OS = Oriental sore. PKDL = Post-kala-azar dermal leishmaniasis. VL = Visceral leishmaniasis.

The New World *Leishmania* species parasitic in man.

Species	Main habitat	Main vertebrate host range	Main vectors	Disease spectrum
L. b. braziliensis	Tropical forest becoming synanthropic?	Edentates? Adapting to dog?	Many suspected vectors	OS, MCL (Espundia)
L. b. guyanensis	Tropical forest	Sloth and other edentates	*Lutzomyia umbratilis*	Pian bois
L. b. panamensis	Tropical forest	Sloths	5 spp. of *Lutzomyia*	OS
L. b. peruviana	Semi-arid highland valleys	Dog, man?	*Lu. verrucarum*? *Lu. peruensis*	OS
L. donovani	Mostly semi-arid tropical zone (expanding)	Domestic dog	*Lu. longipalpis*	Infantile VL
L. mexicana amazonensis	Wet tropical forest (Igapo)	Forest rodents, esp. *Oryzomys* sp., *Proechimys* sp. and opossum?	*Lu. flaviscutellata*	OS, DCL
L. m. garnhami	Cloud forest		*Lu. townsendi*	OS
L. m. mexicana	Humid tropical forest	Forest rodents	*Lu. olmeca*	OS, chiclero ulcer
L. m. pifanoi	?	?	?	DCL
L. sp. (Dominican Republic)	Forest?	?	?	DCL

DCL = Diffuse cutaneous leishmaniasis. Infantile VL = Infantile visceral leishmaniasis. MCL = Mucocutaneous leishmaniasis. OS = Oriental sore.

Figure 10: World Distribution of *Leishmania donovani* (Molyneux and Ashford, 1983).

World Distribution of *Leishmania donovani*

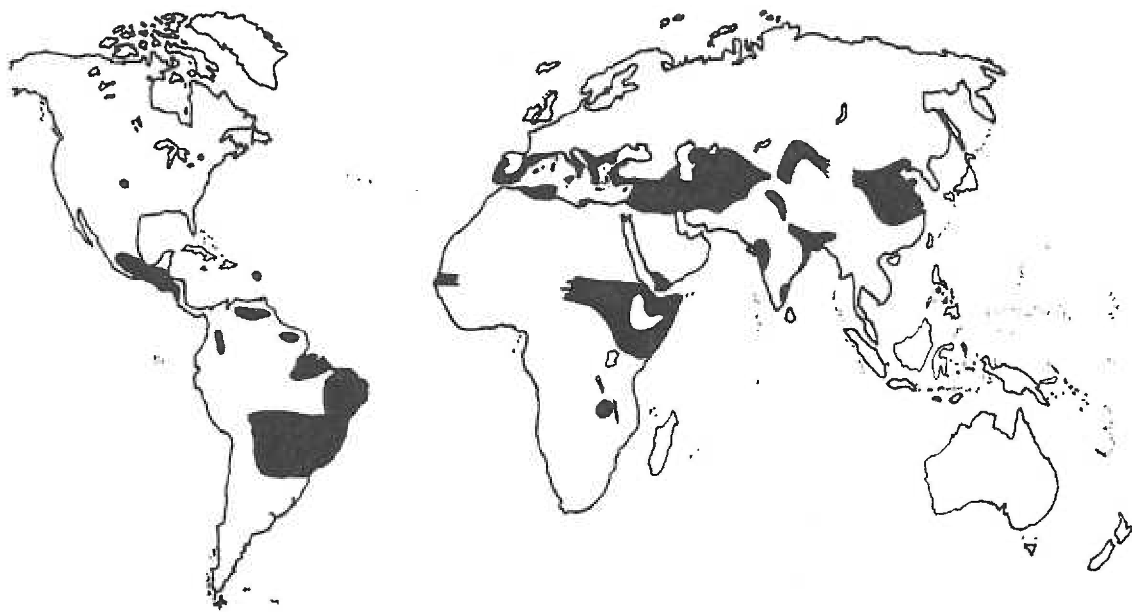
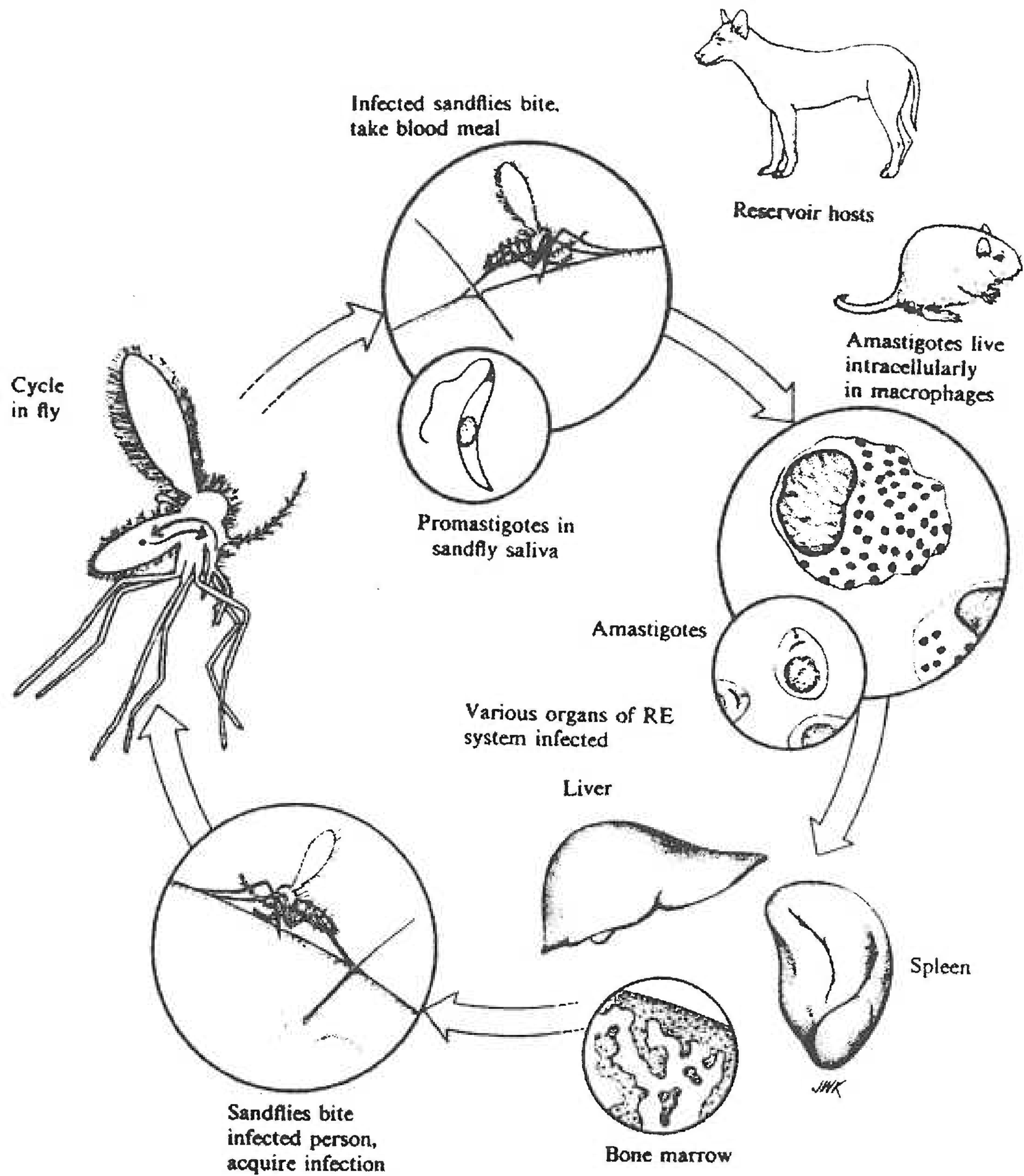


Figure 11: Life Cycle of *Leishmania donovani* (Despommier et al., 1995).

Life cycle of *L. donovani.*



activated within the blood stream. The route of migration to spleenic macrophages, liver Kupffer cells, and the myelocytes of the bone marrow has not been carefully studied and is unknown. Parasite ligands that have been implicated in macrophage uptake identified so far include gp63, LPG and specific flagellar epitopes. They bind to surface macrophage receptors such as Fc, C3b (CR1 and CR3) and fibronectin receptors (Chang, 1979; Bray, 1983; Russell and Talmas-Rohana, 1989; Singla and Vinayak, 1993). Adaptation to the phagosome results in the reduction of the flagellum until it is essentially vestigial within the now amastigote form. As lysosomal vesicles fuse with the phagosomal compartment, *L. donovani* effects it's environment by impairing the normal macrophage oxidative response with LPG (Brandonisio et al., 1993). It modulates the protein kinase C cascade, dampens the ability of the infected macrophage to respond to activating cytokines (Oliver et al., 1992; Moore et al., 1993) and stabilizes it's host by inhibition of apoptosis (Moore et al., 1994a; 1994b). Amastigotes proliferate intraphagolysosomally and eventually infect new macrophages again by phagocytosis. However, this time the parasite produces a large amount of high affinity heparin binding receptors on their surface to utilize the heparin sulfate proteoglycans of the host cell as ligands (Love et al., 1993). The cycle goes full circle when a naive sandfly bites an infected mammal and amastigotes are imbibed. Cell division occurs inside the peritrophic membrane formed by the

abdominal cells of the vector to contain the blood meal. Transformation into flagellated promastigotes occurs and cell division continues for about 7 days in the midgut of the sandfly. Parasites resist excretion by flagellar interdigitation with microvilli. Vector specificity to the various species of *Leishmania* has been found to be directly correlated with LPG terminal polymorphisms and consequent midgut attachment or elimination (Pimenta et al., 1994). If attachment occurs, migration to the mouth parts follow and further transformation to short promastigotes ensues. Finally detachment and movement to the proboscis allows completion of the morphological change to the mammalian infectious form of slender metacyclic promastigote (Tanner, 1996).

2. Visceral Leishmaniasis (Kala-azar): Visceral leishmaniasis or kala-azar is the disease that results from infection with *L. donovani* and may be epidemic, endemic or sporadic. Peculiar characteristics in different locals has justified the classification of visceral leishmaniasis into three categories:

- *African kala azar* is found in the eastern half of Africa from the Sahara in the north to the equator in the south. Sporadic cases have also been reported in west Africa. In this region, the disease primarily affects older children and young adults (10-25 years) and the parasite is endemic in dogs and several wild carnivores in many areas. Resistance to therapy with antimony compounds is more common than in other forms of kala azar

found in the rest of the world.

- *Mediterranean, or infantile, kala azar* is seen primarily in the Mediterranean area, China, Russia and Latin America. It is a disease of children under the age of 4, but adults, particularly travelers to endemic areas, are not spared. Dogs, jackal and foxes serve as primary reservoirs. Sometimes the ***Leishmania donovani Complex*** sub-species of *L. infantum* and *L. chagasi* are responsible.
- *Indian kala azar* has an age and sex distribution similar to African kala azar. Humans are the only known reservoir.

A genetic component of host resistance to infection has been suggested based on the discovery by Bradley and Kirkley (1977) of a single locus called *Lsh* located on chromosome 1 in mice which controls the innate resistance/ susceptibility phenotype. A *Bcg* locus (Vidal et al., 1993) describing the exact phenotype to *Mycobacterium bovis* and *Salmonella typhimurium* infection was determined to be identical to *Lsh* (Vidal et al., 1995).

L. donovani infection triggers both a humoral and cellular response which seldom correlates in intensity or kind with the immunological status of the host. An incubation period of three weeks to eighteen months from infection to disease presentation is common. The onset of disease may be insidious or abrupt with the latter more common to individuals from non-endemic areas. Infections are

characterized by fever with nocturnal spikes similar to those induced by malaria and flu-like symptoms including diarrhea and cough are frequent. Non-tender splenomegaly becomes dramatic by the third month with liver enlargement less conspicuous. Pancytopenia, leucopenia, hypoalbuminemia and polyclonal hypergammaglobulinemia (IgG and IgM) are characteristic. Anemia is multifactorial with contributions from autoimmune hemolysis, splenomegaly, and gastrointestinal blood loss sometimes exacerbated by thrombocytopenia. Edema, cachexia and hyper pigmentation (kala azar means "black fever") are late manifestations. Without treatment death occurs within 3 to 20 months in 90-95% of adults and 75-85% of children, usually from gastrointestinal hemorrhage or superinfections that result following the concomitant significant down-regulation of the host immune system by the parasite. Recovery from visceral leishmaniasis does not seem to afford long-term immunity. However, some reports of protection from clinical cure exist (Manuel and Behin, 1987). Clinically successful cases may develop post-kala-azar dermal leishmaniasis (PKDL) after a 1-2 year latency. Three percent of African cases and ten percent of Indian cases do so. PKDL is characterized by a spectrum of lesions ranging from depigmented macules to wart-like nodules over the face and limbs.

3. Treatment and Control: To ensure a positive diagnosis and to avoid confusion with malarial disease, several methods to determine infection with *L.*

donovani have been developed and can be divided into three categories:

a. Parasitological diagnosis. Parasites visualized by stained biopsy smears of bone marrow, spleen or lymph gland can be identified as round ovoid bodies 2-3 μm in size in the cytoplasm of macrophage cells. To reduce error, cultures should be carried out in parallel. Extreme care must be taken in performing the biopsies as hemorrhaging may result.

b. Serological methods. Specific humoral antibodies are found in cases of visceral leishmaniasis and allow diagnosis utilizing IFAT (indirect fluorescent antibody test), ELISA (enzyme-linked immunosorbent assay) and DAT (direct agglutination test). IFAT detects a very early but short-lived antibody response that becomes negative 6-9 months following cure. One drawback is the difficulty of deciding significant end-point titres and possible cross-reactivity with other trypanosomatidae antigens. The ELISA is a useful field test due to it's ease of completion but problems of high false positives (it does not differentiate between current and past infections) make it less useful than the IFAT or DAT for laboratory diagnosis. No sophisticated equipment or special skills are required for the DAT test which can be performed on serum, plasma, blood spots or whole blood. Most importantly, the reagents are inexpensive and stable making it perhaps the most useful serological test. Standardization of antigen, not commonly commercially available, is most problematic of any of these tests,

resulting in misdiagnosis (false negatives and false poitives).

c. Molecular methods of diagnosis. Although there are no molecular tests routinely used for standard field diagnosis, current research is promising. As more and more species specific DNA sequence becomes known, hybridization, diagnostic PCR and restriction endonuclease methods should become, at least, routine laboratory analytical tools. A limiting factor is cost.

The genomic repeat Lmet is specific for members of *L. donovani* Complex and has been utilized in Brazil to detect parasites in dogs (Howard et al., 1991) and sandflies in both Brazil (Cabral et al., 1993) and Ethiopia (Gebre-Michael et al., 1993).

Treatment of individuals with visceral leishmaniasis is imperative as greater than 90% of unmanaged cases die within one year. In addition to medication, supportive care is essential. Bed-rest, a high protein diet, vitamin supplementation, and, if necessary, blood transfusions are important in recovery (Bosdech and Mason, 1992). As death from visceral leishmaniasis usually occurs through opportunistic infections that thrive largely because of *L. donovani's* immunosuppressive action, antibiotics are usually given for control. The drug susceptibility of *L. donovani* parasites vary in different geographical locations throughout the world (Berman, 1988) yet pentavalent antimonials, used commonly just after the turn of the twentieth century, remain the first line anti-

leishmanial drugs of choice. A thirty day regimen of three daily intravenous injections of sodium stibogluconate or meglumine is recommended as standard protocol (Thakur, 1986). Side-effects include anorexia, nausea, malaise and occasional ECG alterations (Bryceson et al., 1986; Berman, 1988). Allopurinol and γ-interferon therapy as a supplement to sodium stibogluconate has also been recommended (Chunge, 1985; Badaro et al., 1990). Children are successfully treated with a combination of allopurinol and meglumine antimoniate (Di Martino et al., 1990). Pentamidine and amphotericin B are substitutes in cases of drug resistant failures. A prolonged course of antimonials is usually recommended for patients who develop post-kala-azar dermatitis (PKDL) following cure of visceral leishmaniasis (Thakur et al., 1987). Toxicity of these drugs is high and limited alternatives in cases of drug resistance exist. Required hospitalization and injection of the drugs makes discovery of new, effective chemotherapeutic agents of the utmost importance. Development of inexpensive, oral treatments would be extremely beneficial.

Prevention and control of *L. donovani* infections can only be effected by reduction or elimination of the vectors or reservoirs. Mediterranean spraying of DDT to eradicate malaria infested mosquitos significantly altered the sandfly population and as a side benefit, a stark reduction in kala-azar occurred. With DDT no longer in use and no other dramatically effective chemical control

available, *L. donovani* continues to thrive as a human parasite. Spraying insecticide around the windows and doors of homes is somewhat productive as sandflies are relatively weak and rest between short flights or hop from place to place (Despommier et al., 1995). China undertook a radical program to reduce the dog population that served as a reservoir. The success rate was higher in urban than in rural areas and although leishmaniasis still exists, it is diminished in magnitude (Despommier et al., 1995).

Development of a vaccine is seen by some as a slim hope to prevent visceral leishmaniasis yet more hopeful for infections with other *Leishmania* species, particularly *L. major* (Tanner, 1996). A variety of techniques for immunization are currently being attempted (Modabber, 1995). Killed parasites added to BCG as an inoculum; attenuated, live parasites made conditionally viable through targeted gene replacement; cocktails of defined proteins or synthetic peptides, and recombinant bacteria or virions producing specific leishmanial proteins are some of the vaccines under development.

4. Drug Resistance and Future Outlook: The emergence of drug resistance and it's rising prevalence is severely compromising global health programs. Treatment of leishmaniasis includes few choices of chemotherapeutica most of which are moderately to highly toxic, a combination which poses overwhelming obstacles with catastrophic consequences. Resistant *L. donovani*

mutants arise by a number of mechanisms. Target proteins can become modified to reduce drug binding affinity or overproduced to surmount drug pressure. Transport proteins can be affected to reduce drug accumulation or increase elimination. Survival of *L. donovani* is accomplished by undergoing genetic mutations or through DNA sequence amplification (Marr and Ullman, 1995). The latter can occur via two distinct mechanisms. The first is amplification of linear extra chromosomal elements consisting of large linear stretches of DNA (Wilson et al.,1992; Hanson et al., 1992). Expression of circular episomes is the second mechanism of amplification (Beverley, 1991). Both are capable of stabilizing following removal of long term drug pressure. Although the mechanism is not understood, it is generally believed that accumulated centromeres and telomeres provide long term genetic stability of drug resistance.

Metabolic proteins shown to be involved in drug resistance include, DHFR-TS (Ouellette et al., 1990), IMPDH (Wilson et al., 1992) and ODC (Hanson et al., 1992). Two transport genes called *pgpA* (Callahan et al., 1991) and *ldmdrl* (Henderson et al., 1992) confer resistance to hydrophilic and hydrophobic compounds respectively.

The rising tide of resistance makes it imperative that innovative, novel therapies are discovered. An avenue to surmount drug resistance is to have available several efficacious drug choices that can be given in combination.

Primary concerns are compatibility, pharmacodynamics and pharmacokinetics. As investigators continue to unravel the mystery of *L. donovani's* basic biology, the future begins to look brighter. A greater understanding at the molecular level expands the possibilities of therapy and reduces the magnitude of challenge.

C. *Mycobacterium tuberculosis:* *Mycobacterium tuberculosis*, a facultative intracellular prokaryotic bacilli, is the causative agent of the disease tuberculosis (TB). On March 24, 1882 Robert Koch gave his monumental presentation to the Berlin Physiological Society describing the tubercule bacillus, *Mycobacteria tuberculosis,* including convincing evidence that tuberculosis was the disease that resulted from it's infection. Ironically, he had wished to speak to the pathologists but as many great doctors of the time believed that tuberculosis was caused by a tumor or a gland problem and was not contagious, he was barred from doing so. Two earlier scientists, H. Francastoro in 1546 and Benjamin Martin in 1722, had proposed TB to be contagious but to no avail. Koch's technical contributions of staining organisms and capacity to grow *M. tuberculosis* on solid media lead to the subculture of individual colonies, an important capacity for positive identity and characterization. His critical proof of the discovery of *M. tuberculosis* as the infectious agent responsible for TB became widely adopted and known as Koch's postulates. Bacilli were isolated from an animal, grown in pure culture, reintroduced into another animal and the

same morbid conditions arose (Koch 1932). His work revolutionalized scientific thinking and established microbiology as a discipline and hence, he is known as the "father of microbiology." Interestingly, another scientist who would become well known for his life's devotion to parasitic diseases, Paul Ehrlich, was present at Koch's talk. He developed improved staining techniques and found acid-fast bacilli in his own sputum, thereby establishing his self-diagnosis (Daniel et al., 1994).

Tuberculosis has plagued humans for several millennia and is currently identified as the world's foremost cause of death from a single infectious agent (Shinnick, 1996). Approximately one-third of the world's population, or roughly 1.9 billion people, are or have been infected and of these, almost three million die each year. Deaths due to TB among persons age 15-59 is 20% worldwide! Eight to ten million new cases are reported each year. It is a devastating disease particularly in developing nations where 95% of infected persons reside. Predictions have been made that unless a concerted global intervention is undertaken soon, the current situation considered serious in magnitude will deteriorate rapidly (Snider et al., 1994).

Skeletal remains indicate a long history for tuberculosis. Before 8000 B.C. humans are believed to have been mostly wanderers and TB seems to have occurred only sporadically (Clark, 1962). With the development of primitive

agriculture and the domestication of animals came settlement in dwellings. The first endemic infection of humans may actually have come from *M. bovis* acquired by individuals with living quarters over their stables, a common practice for providing heat. As communities continued to enlarge, environmental changes may have caused a shift in the fragile balance between the bacterium and it's host. The most widely accepted theory explaining the expansive epidemic and subsequent decline of TB is given by Stead (1992). Generation time is comparably short for the bacterium (<1 day) compared to the human host (close to 20 years). Mutation rates can more rapidly effect an evolutionary adaptation in the bacteria long before it's host. Consequently, many susceptible members of the human population are eliminated before their genes are passed on. Over time resistance increases and after successive host generations a once devastating disease becomes less and less so. England saw a sharp rise in TB incidence in the early 1600's and over the next two hundred years it magnified to epidemic proportions (Castiglioni, 1933; Webb, 1936; Cummins, 1949; Dubos and Dubos, 1952) and spread throughout Western Europe. Even considering the feudal times and the increased death rate due to war, it is estimated that one in four deaths resulted from TB. As a repercussion of infected Europeans colonizing distant lands, TB had spread in full epidemic force to Eastern Europe, Asia, Africa, North and South America by the 1800's (Diamond, 1992). The island population of

New Guinea was initially exposed to TB in the early 1950's where it produced typhoid-like symptoms typical for susceptible individuals not previously exposed (Brown et al., 1984). The epidemic peak in any particular geographical location is estimated to have occurred 50-75 years after it's onset followed by a slow decline as more resistant survivors reproduce. A similar pattern is seen in the United States as currently 90% of infected individuals remain asymptomatic (Comstock, 1982). Other serious complications such as HIV co-infection, alcoholism, chronic renal failure, diabetes mellitus, drug abuse, and crowded or unsanitary living conditions are often present in modern day cases of TB.

Common sense regulations were passed and coughing and spitting in public became unacceptable. Quarantine to sanatoriums was common. Hospital linens were often burned to prevent spread of TB infections.

In the 1930s William Wells began a series of studies involving the airborne nature of the pathogen. A student of his, R.L. Riley, completed a classic study of animal exposure to tuberculosis hospital ward air that had been delivered either untreated or UV irradiated. No infections occurred in the guinea pigs exposed to treated air while a rate of over 50% was observed in the group subjected to untreated air (Riley et al., 1962). The most dramatic change in the outlook of tuberculosis came in the 1940s and early 1950s when Selman Waksman (discovered in 1944, published: Pfuetze et al., 1955) and Robitzek and Selikoff

(1952) demonstrated the tuberculocidal effects of streptomycin and isoniazid respectively. The era of successful chemotherapeutica began and the end of tuberculosis seemed in hand. And until recently, most believed TB to be curable.

1. Life cycle of *Mycobacterium tuberculosis:* Ah-chew! A sneeze from an infected individual spews out millions of particles averaging 10 μm in diameter containing disease causing bacilli. A cough or just speaking can do the same. The particles form droplet nuclei and evaporate only until equilibrium with atmospheric pressure occurs. They remain suspended for extremely long periods of time having settling velocities of approximately 10 mm/min and can be continuously resuspended by air convection allowing ample opportunity for inhalation by a new host (Smith and Moss, 1994). Ingestion is also an effective route for infection but at a 10,000 fold reduced rate (Gaudier and Gernez-Rieux, 1962).

Ninety percent of the newly inhaled *M. tuberculosis* organisms will settle in the upper respiratory epithelium and be expelled by the action of mucocillia (Nardell, 1993). Those that make it deep into the lung either invade or bind to alveolar monocytes and macrophages and undergo phagocytosis. The evidence for invasion consists of a single convincing report that a cloned fragment of *M. tuberculosis* DNA conferred invasiveness to *E. coli* (Arruda et al., 1993), thereby insinuating that in addition to the generally accepted mode of entry, phagocytosis,

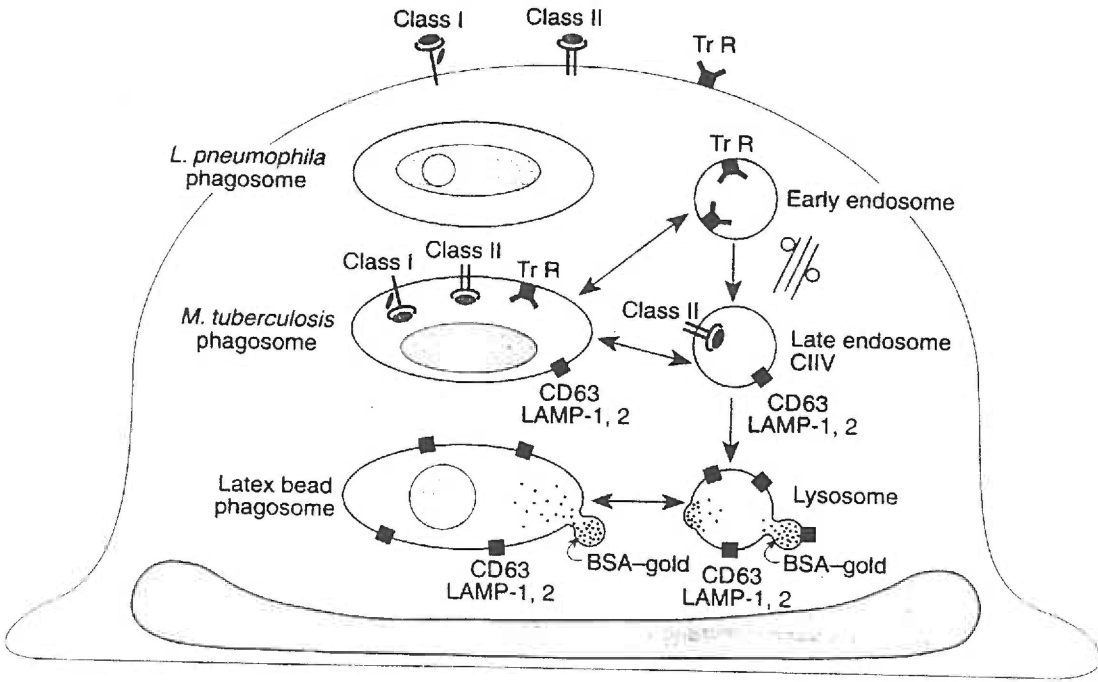
M. tuberculosis also has an inherent ability to actively invade human cells.

Phagocytosis is initiated by binding of the bacteria to complement and mannose receptors. CR1 and CR3 appear to be the major monocyte receptors while CR4 (Hirsch et al., 1994) and especially mannose receptors play the significant role in macrophages (Schlesinger et al., 1993). Once the alternative complement pathway is activated by cord factor (a surface glycolipid trehalose dimycolate) (Ramanathan et al., 1980), C3 protein probably serves as opsins for the complement receptors while binding to the macrophage mannose receptors occurs via interaction with the terminal mannose residues of the LAM coat of *M. tuberculosis* organisms (See Figure 12) (Schlesinger et al., 1994).

Once inside their intracellular niche, two outcomes are possible. The bacteria either are killed or affect their environment and survive. Survival includes slow multiplication (approximately 24 hr doubling times) and procession to a disease state or the capacity to lie "dormant" for years in wait for a dimunition in immune competency. Killing of *M. tuberculosis* has been postulated to occur in numerous ways and the literature is riddled with confusion. In fact, there is literally no conclusive direct evidence that human macrophages can kill *M. tuberculosis* (O'Brien et al., 1996). It is all circumstantial, mostly inferred from work on *M. avium* or *in vitro* data collected using animal models. Unfortunately, there is not a perfect animal model. Either the pathology or the immunology of

Figure 12: Receptor-ligand Mediated Phagocytosis of *M. tuberculosis*

(Schlesinger, 1996)


Class I
Class II
Tr R
L. pneumophila phagosome
Tr R
Early endosome
Class I
Class II
Tr R
M. tuberculosis phagosome
Class II
Late endosome CIIV
CD63 LAMP-1, 2
CD63 LAMP-1, 2
Latex bead phagosome
Lysosome
CD63 LAMP-1, 2
BSA–gold
CD63 LAMP-1, 2
BSA–gold

infection is different from humans, leaving an imposed compromise regardless of the system chosen. And although parallels surely can be made, *M. avium* is not *M. tuberculosis* as marked differences in virulence exist.

Macrophages have a large repertoire of general antimicrobial mechanisms which may also be active against *M. tuberculosis*. They can be classified as either oxygen-dependent or oxygen-independent (Lowrie and Andrew, 1988). Phagocytosis not involving the alternate complement pathway is accompanied by a respiratory burst.

NADPH oxide catalyzes a one-electron reduction of molecular oxygen to superoxide. Further interactions can yield the formation of damaging amounts of peroxide, hydroxy radicals and singlet oxygen (Halliwell and Gutterridge, 1984). Clearly the bulk of evidence indicates that respiratory burst products are not necessary nor sufficient for killing *M. tuberculosis*, yet they play an important role, merely not an exclusive role.

That macrophages utilize several oxygen-independent systems to illicit microbialcidal effects is well established. Less clear is which of these mechanisms are active against mycobacteria. They include, but may not be limited to, lysosomal action, antimicrobial proteins and peptides, sequestration of iron, and metabolism of the amino acids, ornithine, arginine and tryptophan.

Armstrong and Hart (1971) first reported that living *M. tuberculosis*

organisms do not reside in lysosomes while phagosomes containing dead *M. tuberculosis* bacteria were lysosomal-like, implying a killing nature of lysosomal fusion. In fact, extracts from lysosomes do kill *M. tuberculosis in vitro* (O'Brien et al., 1996).

Peptide and protein antibiotics are part of the defense mechanism of neutrophils, macrophages and intestinal epithelium (Spitznagel, 1990; Selsted et al., 1992; Lehrer et al., 1991;1993) which contain defensins, cathepsin, azurocidin, indolicin (Delsal et al., 1992) and bactencins (Frank et al., 1990). The defensins HNP 1, 2 and 3 are equally active against *M. avium* but untested against *M. tuberculosis* (Ogata et al., 1992). The significance of these molecules remains unsolved.

Iron is necessary for life so it is not surprising that macrophages would attempt to sequester iron as a means to kill invasive organisms. It is believed that macrophages may ingest neutrophils to obtain increased levels of lactoferrin and transferrin. This mechanism seems effective against *Legionella pneumophila* (Byrd and Horwitz, 1993). However, the influence of an iron chelator depends on its degree of saturation. *M. tuberculosis* exochelins seem to have a strong capacity to aquire iron regardless of host cellular attempts to sequester it.

The metabolism of the amino acids ornithine, tryptophan and arginine by macrophages as mechanisms of microbialcidal action has recently been receiving

the most attention. No doubt the most exciting is that of arginine conversion to reactive nitrogen oxides. First, let us consider tryptophan actions. INF-γ induces the enzyme indolamine 2,3-dioxygenase which catalyzes the conversion of tryptophan to N-formylkynurenine (Byrne, 1987) thereby inhibiting growth of several intracellular pathogens including *L. donovani* (Murray et al., 1989) simply by depleting tryptophan, apparently an essential nutrient. Autocrine production of picolinic acid, a final product of tryptophan metabolism, influences antimicrobial action by synergism with INF-γ in the activation of macrophages (Melillo et al., 1993).

As mentioned earlier, ornithine metabolism to polyamines had long ago been shown to be antimycobacterialcidal (Hirsch and Dubos, 1952). To avoid redundancy it will not be discussed further here. A renewed interest in this area of research may prove fruitful.

Arginine metabolism as a source of reactive nitrogen oxides poses another source of controversy in the mycobacterium field. The discrepancy arises from conflicting reports that TNF-α, GM-CSF (granulocyte/macrophage colony stimulating factor) and INF-γ activated macrophages either utilized an RNI killing mechanism (Denis, 1991) or showed that the anti-*M. avium* activity was RNI (reactive nitrogen intermediate)-independent (Bermudez, 1993). The issue has not been satisfactorily resolved (O'Brien et al., 1996). That ·NO (nitric oxide free

radical) can be produced from arginine by human macrophage-inducible nitric oxide synthetase is well accepted (Review by Knowles and Moncada, 1994). Peroxynitrite ($OONO^-$) can be formed through the interaction of superoxide anion (Halliwell and Gutteridge, 1992) or hydrogen peroxide (Noronha-Dutra et al., 1993) and $^{\cdot}NO$. The latter reaction also produces singlet oxygen (Noronha-Dutra et al., 1993). Decomposition to hydroxyl radical ($^{\cdot}OH$) and nitrogen dioxide radical ($^{\cdot}NO_2$) occurs (Halliwell and Gutteridge, 1992). All of these molecules are powerful oxidants. If and when they are produced in response to mycobacterial infection remains to be conclusively proven, which may be an extremely difficult task.

At this time we are left with the fact that humans are certainly capable of overcoming the blight of mycobacteria through a killing action. Several mechanisms may play a role just as they do for other bacterial infections. What mechanisms are important or actually function against these unique organisms is often hotly contested (O'Brien et al., 1996). Ultimately, technical difficulties must be overcome to discover the answers. As this occurs, exciting pieces of a very complex puzzle will fall into place.

Adaptation to a harsh fate is necessary for survival of any intracellular parasitic organism and *M. tuberculosis* is no exception. Evidence from several labs indicate that upon phagocytosis, this bacteria is very capable of modifying

its environment to suit its specific needs (Clemens and Horwitz, 1995; Xu et al., 1994; Sturgill-Koszycki et al., 1994; McDonough et al., 1993). Acidification of the phagosome is mild (pH = 6.0-6.5), probably due to the combination of exclusion of the vacuolar proton pump (Xu et al., 1994), release of ammonia (Gordon et al., 1980), and the possible retainment of Na/K ATPase (Clemens, 1996). A relatively slow clearance of MHC class I molecules is followed by a persistence of MHC class II molecules on the phagosomal membrane (Clemens and Horwitz, 1995). Coupled with the fact that transferrin receptor is also present, these data indicate a presumable lack of maturation and concomitant fusion with a lysosomal compartment, as well as a continuing interaction of *M. tuberculosis* phagosomes within the endocytic pathway (Clemens, 1996).

Although the life cycle of *M. tuberculosis* bacilli is becoming more deeply understood, several questions remain unanswered. How do initial infections resolve without chemotherapy? What are the particular "killing" factors? What is/are the specific factor(s) that control the inhibition of phagosome-lysosome fusion? What are the characteristics of the phagosomal membrane? Does *M. tuberculosis* depend on endosomal delivery of nutrients or are sufficient transporters available? Is their presence regulated in any way by the bacteria? What allows the mycobacteria to lie dormant for years and how can this regulation be disrupted? How accurately do studies involving cell culture or animal models

depict infections in humans? And, ultimately, how can we, as scientists and clinicians, obtain answers to these difficult questions?

2. Tuberculosis: Tuberculosis is the disease that results from infection with *M. tuberculosis.* General symptoms include fever, malaise, night sweats and weight loss (Peloquin and Berning, 1994), manifestations thought to be mediated by cytokines, particularily TNFα (Takashima et al., 1990). Presentation of the disease can be either pulmonary or extrapulmonary. Of infections not involving HIV complications, eighty-five percent are localized to the lungs resulting in cough, chest pain and dyspnea (difficulty in breathing). A physical examination in a clinical setting is not always productive in defining TB. Rales may be heard in the area of involvement and broncial or amphoric breath sounds could respectively indicate lung consolidation or a cavity. More definitive is a radiographic examination as pulmonary TB commonly produces an aberrant chest film. Approximately 15% of TB cases involve extrapulmonary sites which are more diagnostically challenging due to reduced physician familiarity and inaccessibility of sites involved. A multiplicity of extrapulmonary disease can occur including: lymph node, plural, genitourinary, skeletal, central nervous system, abdominal, pericardial as well as miliary (disseminated) TB.

The epidemiology and pathogenesis of TB is changed upon co-infection with HIV and vise-versa. Either one enhances the probability of disease

progression with the other. For HIV-TB cases, a sharp reduction in life expectancy is predicted. TB naturally activates macrophages that can also harbor HIV and leads to increased expression of virus and earlier progression to full observation of AIDS. HIV can lower CD4+ cell count and the resulting immunocompromise limits ones ability to contain *M. tuberculosis* infection. The rate that active TB develops in HIV-TB infected patients is astoundinding! A rate of approximately **10% per year** compares to a general risk of **8% in a life-time** for healthy individuals (Sepkowitz et al., 1995). Another complicating factor is that co-infection with HIV drastically changes the clinical presentation of TB. Rather than a predominance of pulmonary involvement, disseminated disease is more common yet atypical in nature. Presumably the immunocompromised patient is incapable of encapsulating foci and the result is a diffuse uniform pattern of lymphocytic infiltration and edema instead of contained 1-2 mm "miliary" nodular granulomas. Although disseminated disease usually includes lung involvement, sputum samples may be negative whereas blood and bone marrow cultures are more likely to indicate TB infection. Anergy (immunological hyporesponsiveness) is common, making the PPD skin test an ineffective tool. Radiographic results seem to be dependent upon the patient's degree of immunocompromise (Hopewell, 1994).

3. Treatment and Control: Before recommendations for treatment can

be properly determined, a positive *M. tuberculosis* identification must be made. The general nonspecific symptoms of TB make substantiation somewhat challenging and further complications arise when the effectiveness of the diagnostic PPD test is considered. The Mantoux test involves the intracutaneous injection of 5-tuberulin units of PPD on the volar aspect of the forearm resulting in a small raised wheal. Forty-eight to seventy-two hours later an experienced professional checks the site for induration. A >10 mm and >5 mm area indicates a positive TB test for immunocompetent and HIV infected patients, respectively (Centers for Disease Control, 1992). A negative test is common in HIV infected individuals and is considered meaningless while a positive test indicates any of three possibilities: active infection, prior exposure or vaccination. The test is somewhat more reliable in non-HIV positive individuals. However, the rate of anergy is also quite high at about 25% (Nash and Douglas, 1980).

A chest x-ray is a standard diagnostic tool for pulmonary involvement of TB but has limitations as well. As many as 20% of HIV-TB cases may yield normal radiographs (Sharp et al., 1993). Bacteriologic and histologic examinations are the next step. Unfortunately, 50% of HIV-TB co-infections result in acid-fast negative sputum smears (Schluger and Rom, 1994), a common initial step in diagnosis. Fortunately, positive identification of *M. tuberculosis* no longer depends on biochemical tests such as production of niacin, catalase, and

nitrate reductase from 2-3 week cultured bacteria. Magnetic polystyrene microspheres coated with specific antibody can be added to samples to enhance recovery via a magnet (Murtagh et al., 1995). This technique improves smear detection as well as increases sample concentration for culture or other diagnostics. Recent molecular biological innovations have made it possible to rapidly identify the presence of *M. tuberculosis* as well as specifically discriminate it from other mycobacteria (Marshall and Shaw, 1996). Techniques utilizing PCR are advantageous in that culture is unnecessary. Time required to culture *M. tuberculosis* from samples is usually 2-3 weeks. PCR relies on amplification of small amounts of DNA isolated from as little as 10-1000 organisms in a sample and can be processed in a few hours (Brisson-Noel et al., 1989). The most commonly used target is the insertion sequence IS6110, a high copy (up to 20), specific DNA region for *M. tuberculosis*.

Sensitivity, specificity and positive predictability values for PCR are acceptible, usually between 83%-95% (Clarridge et al., 1993). Contamination of samples and the presence of inhibitors to the PCR reaction are the most prevalent problems. Non-PCR-based exponential nucleic acid amplification methods are now available. Transcription-mediated-amplification (TMA) is carried out in a single test tube and detects specific *M. tuberculosis* rRNA with an acridinium ester-labelled DNA probe (Jonas et al., 1993). A kit is available through Gen-

Probe (San Diego) and can be essentially completed in 4 hours. Strand displacement amplification coupled with fluorescence polarization (SDA-FP) monitors the conversion of a fluorescently labelled oligonucleotide probe from the single to double strand form in positive samples (Walker et al., 1996). Both of these methods increase sensitivity and reduce the possibility of contamination as they are self-contained throughout the process time. Other new technologies are available and have been reviewed recently (Sepkowitz et al.,1995; Marshall and Shaw,1996).

M. tuberculosis drug resistance considerably complicates the treatment of tuberculosis. Standard practice today is to prescribe treatment on an individual basis following the results of susceptibility testing. Three and four drug regimens given for 6 to 9 months or longer are generally required. Meningitis, osteomyelitis and miliary tuberculosis frequently demand protracted treatment (Barnes and Barrows, 1993). First-line TB therapy consists of five medications (Gilman, 1990) including isoniazid (INH), rifampin, pyrazinamide (PZA), ethambutol, and streptomycin while second-line drugs, used in areas of high resistance, include the floroquinolones (ciprofloxacin, ofloxacin, and it's L-isomer, levofloxacin), cycloserine, capreomycin, ethionamide, para-aminosalicylic acid and rifabutin.

Although INH is the mainstay of TB therapy, liver toxicity and peripheral neuropathy are common side-effects (Sepkowitz et al., 1995). Rifampin, equally

as potent as INH, is also hepatotoxic and together the toxicity is additive rather than synergistic (Steele et al., 1991). Hyperuricemia and hepatotoxicity result from PZA yet its overall effectiveness has led to an increase in useage (Sepkowitz et al., 1995). Regular screening of color vision for detection of optic neuritis allows alterations in therapy and subsequent prevention of this feared side-effect (Sepkowitz et al., 1995). Renal failure may also occur. Common resistance and the need for intravenous or intramuscular administration limit the use of streptomycin, the first curative agent found against tubercuosis. Ototoxicity and renal dysfunction are side-effects.

Manifestation of second-line drug toxicities consists of fever, rash, agitation, and gastrointestinal disturbances among the fluoroquinolones. Central nervous system abberations ranging from somnolence to seizure may arise during cycloserine treatment and sometimes can be abbrogated through pyridoxine (Vitamin B_6) administration. Capreomycin causes otic and renal toxicity and must be injected, limiting its ease of use. Doses of ethionamide are increased slowly according to gastrointestinal tolerance. Severe gastrointestinal upset is illicited during PAS therapy, as well as hepatitis and fluid retention. Uveitis develops in some patients treated with rifabutin (Sepkowitz et al., 1995).

The major effort to prevent TB has been through a massive worldwide vaccination effort with bacillus of Calmette and Guérin (BCG). It is the most

common immunization in the world with more than 1 billion children in 182 countries having been treated since the immunization of the first newborn in July of 1921 (Huebner, 1996). Avirulent BCG was developed by Calmette and Guérin after 13 years and 230 consecutive subcultures of a virulent strain of bovine *M. tuberculosis,* originally isolated by Nocard from a cow with mastitis (Gardner 1932). Since that time hundreds of "daughter" strains have given rise to various differences in vaccine characteristics. Efficiency studies have caused considerable controversy. Reports vary from descriptions of literally no protection to almost complete protection. Analysis considered most reliable showed significance with at least three factors: 1) Vaccine efficacy increases with decreasing age at the time of administration. 2) More protection is afforded in areas with a high incidence of TB. 3) Most suprisingly, higher protective effects were seen as distance from the equator increased (Huebner, 1996). The United States government does not mandate BCG vaccination in favor of not comromising the PPD test as a diagnostic tool.

Further effort to improve control of TB involves implementation of programs for direct observed therapy (DOT). Noncompliance (discontinuance or reduction of dosage for one or more chemotherapeutica) of treatment is the most significant factor in the increasing tide of drug resistance (Gostlin L., 1993). Supervised dispensement and individual surveillance of medication compliance

significantly reduces production of resistant *M. tuberculosis* in individuals (Weis et al., 1994). Cost is easily justified if the exponential expense of increased exposure issues are included as a factor (Centers for Disease Control, 1990). The longterm treatment protocol make compliance difficult and non-compliance can have drastic consequences. For example, in 1990, a single individual hospitalized several times for TB complications, took medications while interned but never following discharge. He developed multi-drug resistant (MDR) TB and subsequently infected 9 family members. The total cost of treatment for all 10 individuals was $950,433.

Prophylaxis studies have indicated that high compliance and a favorable risk-benefit ratio with some therapies result in significant prevention of TB occurrence (Centers for Disease Control, 1990). High-risk individuals such as HIV positve patients and persons with a known exposure (health care workers, prison inmates, family members of the infected) may greatly benefit. Prevention of active TB has obvious benefits such as a reduction in the exponential exposure effect resulting in a lessened burden on society as a whole.

4. Drug Resistance and Future Outlook: Emergence of drug resistant tuberculosis is a simple evolutionary phenomenon with a potential for an enormous catastrophic effect on society. Human societal failures such as noncompliance (Goble et al., 1993) and inappropriate physician prescription

(Hahmoudi and Iseman, 1993) have selected for survival of drug-resistant mutants. As a consequence, rates of resistance have risen dramatically and in many areas of the world are above 30% (Iseman and Sbarbaro, 1992, Freiden et al., 1993). Evidence suggests that resistance arises only through random mutations in *M. tuberculosis*. No alternative mechanisms have ever been described.

The mutation rate in *M. tuberculosis* is approximately one in 10^5 - 10^8. As even extensive bacteremia is estimated to result in fewer than 10^9 bacilli present in a patient (David and Newman, 1971), spontaneous multi-drug resistance would be improbable. Therefore, resistance most probably compounds sequentially and clinical evidence suggests that this is the case. Streptomycin treatment alone resulted in some therapeutic failures and concurrent production of resistant bacteria (Mitchison, 1950). As more treatments became available, it was quickly realized that simultaneous drug treatment prevented emergence of resistant mutants (American Thoracic Society, 1986). Unfortunately, even though MDR-TB is preventable, potentiation is currently a fact. The implications are profound: 1) Prophylactic treatment could be rendered ineffectual. 2) Extended therapy and increased management will drive costs of treatment to unreachable levels for impoverished regions of the world. 3) Mortality and morbidity will expand.

Ironically, drug-resistance has been used as a genetic tool to determine the

modes of action for many TB drugs which have historically been derived empirically or serendipitously (Heym et al., 1996). Susceptibility testing has also been advanced. Novel genotype-based techniques are now available (Telinti and Pering, 1996). Automated-PCR-based sequencing (Kapur, 1995), single-strand conformation polymorphism (SSCP) (Delgado and Telenti, 1996), dideoxy fingerprinting (Felmlee et al., 1995), solid phase hybridization (Saiki et al., 1989) and heteroduplex formation (Williams et al., 1994) can be used to determine known resistances and can be utilized to make therapeutic recommendations in a short time course. Figure 13 indicates the molecular targets of resistance for several TB drugs.

Finding solutions to the human problems that *M. tuberculosis* causes will be a challenge. The scientific community needs to rapidly gain knowledge of biochemical and molecular information for this pathogen.. Acquistion of this knowledge is met with considerable obstacles, the most obviousis the inherent danger of culturing *M. tuberculosis* in the lab. Hospitals need more space to accomodate and isolate patients with TB. Clinicians need to implement tighter control over contagious patients through education and possibly legislation. Inadequate therapy no longer poses a threat only to the specific individual but to society at large.

Figure 13. Table of molecular targets of drug resistance in *M. tuberculosis.*

(Telenti and Persing, 1996)

Molecular Targets of Drug Resistance:

Antimycobacterial agent	Gene(s)	Gene product	Frequency of mutations associated with resistance	Commentary
Isoniazid	(i) *katG* (ii) *inhA* /*mabA* (iii) *ahpC*	(i) Catalase-peroxidase (ii) Analogs of *E. coli* fatty acid biosynthesis enzymes EnvM and Fab G, respectively (iii) Alkyl hydroperoxide reductase C	(i) 47-58 % (ii) 21-28 % (frequently in association with *katG* mutations) (iii) 10 %	The relative contribution of defined mutations to a resistance phenotype has not been established. Mutations in *inhA/mabA* generally associated with low-level INH resistance (MIC < 1 μg/ml) and cross-resistance to ethionamide. Targeting KatG codon 315, the putative regulatory region of *inhA*, and *ahpC* may be sufficient to detect close to 90 % of clinical INH-resistant isolates. Additional unknown mechanisms may account for < 10 % of clinical resistance.
Rifampin	*rpoB*	β subunit of RNA polymerase	96-98 %	Mutations occur within a 81-bp core region (codons 507 to 533). Detection of RMP resistance may serve as surrogate marker for diagnosis of MDRTB.
Streptomycin	(i) *rpsL* (ii) *rrs*	(i) ribosomal protein S12 (ii) 16S rRNA	(i) 52-59 % (ii) 8-21 %	Mutations of *rpsL* result in substitution at codon 43 or more rarely, 88. Changes in 16S rRNA involve nucleotides 491, 513, 516, or 903. Additional unknown mechanisms may account for 24-40 % of clinical resistance.
Ethambutol	*embA* *embB*	—	—	Putative operon involved in the arabinogalactan and lipoarabinomannan biosynthesis.
Fluoroquinolones	*gyrA*	DNA gyrase subunit A	75-94 %	All known GyrA mutations among different bacteria can be identified by targeting amino acid region 74 to 113. Additional unknown mechanisms may account for up to 25 % of clinical resistance.

D. ***Mycobacterium avium:*** *Mycobacterium avium* is an avian tubercle bacilli that, upon infection, causes contagious tuberculosis in domestic and wild birds (Thoen, 1994). *M. avium* was previously thought to be of little or no health significance; however increasing pathogenicity in humans and their domestic and captive animals has been reported (Grange, 1991, Thoen, 1990, 1992, 1993).

1. An Opportunistic pathogen in immunosuppressed patients: Long recognized as an uncommon cause of pneumonia in patients with chronic lung disease (Wolinsky, 1979) and childhood localized cervical lymphadenopathy (Pozniak et al., 1996), *M. avium* complex (MAC) is now more frequently seen in disseminated nontuberculous mycobacterial disease of AIDS patients (Horsburgh, 1991). Unlike *M. tuberculosis*, whose presence is indicative of disease, *M. avium* has been well characterized to colonize without causing active illness. For this reason it may have been underestimated as a pathogen (Iseman, 1985). Today, disseminated MAC is the most common systemic bacterial infection in HIV-infected persons and the second most frequent overall opportunistic pathogen in this population (Katz, 1994; Kemper 1994).

Infection is acquired by respiratory or gastrointestinal exposure to the ubiquitous environmental pathogens. Water, food, soil and animals are all considered possible sources (Goslee and Wolinsky, 1976; Messner and Anz, 1977; Wolinsky and Rynearson, 1971). Presumably, the organisms quickly pass

into the blood stream and disseminate. Active disease usually occurs only in individuals with $CD4^+$ cell counts below 50 cells/μl (Chin et al., 1994). General wasting and severe anemia are the most common, yet nonspecific, clinical presentations of disseminated MAC disease. Consistent symptoms include persistent fever, fatigue, night sweats, anorexia, abdominal pain, and chronic diarrhea (Chin et al., 1994). Hepatosplenomegly, lymphadenopathy, and leukopenia can also be seen. MAC infection is intracellular and can involve almost any organ. Since there is a predilection for macrophage infection, the reticulo-endothelial system is affected most frequently but the lymph nodes, liver, spleen, bone marrow, GI tract, brain, meninges, adrenal and thyroid glands, pancreas, kidney, heart and lungs are all common sites of infection. An extraordinarly high concentration of organisms (10^{10} cells/ organ) give a characteristic yellow discoloration to affected areas (Kreher and Zurlo, 1994) leading to significant reduction in AIDS patient life-span. Diagnosis is commonly provided from blood samples and and is similiar to that of *M. tuberculosis*. The two ultimately need to be distinguished from each other for appropriate treatment recommendation (Kreher and Zurlo, 1994).

2. Treatment and Control: Treatment of disseminated MAC in AIDS patients is somewhat similar to treatment of TB; however, isoniazid is usually avoided and *M. avium* effective macrolides (clarithromycin, and sometimes

azithromycin) substitute as the foundation of therapy (Sesin et al., 1996). To lessen resistance development, usually in addition to clarithromycin, at least one other antimicrobial agent is given, either: ethambutol, rifampin or rifabutin, cloflaimine, ciproflaoxacin, ofloxacin or amikacin. Adverse side-effects are often nausea, vomiting, abdominal pain, taste perversion and, as mentioned for TB treatment, systemic toxicities predominate.

Avoidance of MAC exposure for AIDS patients or persons with iatrogenic immune deficiency is nearly impossible as its ubiquitous nature makes even drastic measures (such as boiling all drinking water and completely cooking food) insufficient. Consequently, prophylaxis is an important issue. Currently the U.S. Public Health Task Force recommends rifabutin for HIV-infected patients with $CD4^+$ cell counts below 100cells/µl, and recently clarithromycin received FDA approval for prophylaxis as well. Determination that the patient is free of MAC infection at the initiation of prophylaxis is imperative for resistance prevention, as few drugs are available for treatment of active disease (Sesin et al., 1996). Controlled studies indicate that utilizing preventative measures against MAC in HIV-patients results in long-term benefits in longevity and quality of life.

3. Drug Resistance and Future Outlook: *M. avium* is inherently more drug resistant than *M. tuberculosis* due to greater impermeability of the cell envelope (Suzuki and Inamine, 1994). Resistance that arises from genetic

modifications is similar to that of *M. tuberculosis* (Meier et al., 1994) except for its potential for increased prevalence. Resistance arises from random mutations that occur at a rate of 1 in 10^4 - 10^8. Unlike TB, where whole body bacilli counts may reach only 10^9 cells (David and Newman, 1971), disseminated MAC can result in **a single organ containing 10^{10} bacteria** (Kreher and Zurlo, 1994) with whole body counts considerably higher. The net result is that drug resistant TB develops sequentially while a given population of MAC could become multidrug resistant (MDR) within an individual. The consequences, of course, are vastly different. TB is a communicable disease and MDR-TB can be spread within the general population while becoming more and more drug resistant. The likelihood of MDR-MAC doing so is extremely minimal.

THESIS RATIONALE

The current arsenal of drugs used to treat the three intracellular pathogens on which my thesis project focuses, *Leishmania donovani, Mycobacterium avium,* and *Mycobacterium tuberculosis*, is far from ideal. The available chemo-therapeutica are limited in scope and have been shown to be moderately to highly toxic and possibly carcinogenic. Drug resistance is becoming an increasingly prominent problem. Scientists, clinicians and public health officials all recognize the need for new, more efficacious therapies requiring shorter, easier and more cost effective treatment.

The purine salvage pathway has stimulated considerable therapeutic interest among a spectrum of diseases as its phosphoribosyltransferase enymes are capable of trapping membrane-permeable purine nucleobases intracellularly as phosphorylated nucleotide products. The hypoxanthine-guanine phosphoribosyl-transferase subversive substrate, allopurinol, has been shown to be effective against the Trypanosomatidae family of parasites, including *L. donovani* (Marr, 1991) and is considered a prototype for this paradigm. The intracellular macrophage location of *Leishmania donovani, Mycobacterium avium,* and *Mycobacterium tuberculosis* limits therapeutic posibilities. Purine analogs, which are already used to treat a great variety of human diseases, represent a potentially useful class of compounds for treatment of these refractory infections. In

particular, they may be a logical circumvention to specific problem of drug accessibility.

Characterization of the HGPRT and XPRT purine salvage pathway enzymes is the initial step in the validation of their potential as therapeutic targets in these three pathogenic organisms. Ultimately, long-term goals of utlizing the molecular anatomy of each enzyme for a computational approach to structure-based drug design can be met. The development of effective analogs will be greatly facilitated by the ability to purify large quantities of recombinant protein for biochemical, structural and drug screening studies.

SPECIFIC AIMS

1. **To clone and sequence the *xprt* cDNA from *L. donovani*.**

2. **To clone and sequence the *xprt* gene from *L. donovani***

3. **To clone and sequence the *hgprt* genes from *M. avium* and *M. tuberculosis*.**

4. **To produce the recombinant *L. donovani* XPRT and *M. avium* and *M. tuberculosis* HGPRTs in *E. coli*.**

5. **To purify the respective enzymes to apparent homogeneity and characterize their kinetic parameters.**

6. **To initiate crystallization protocols.**

7. **To complete *in vitro* purine analog drug screens.**

MATERIALS AND METHODS

Chemicals, materials and reagents: *Mycobacterium avium*, isolates 101, 724 and *Mycobacterium tuberculosis*, Erdman strain were kindly provided by Dr. Ronald Barry, V.A. Medical Center, Portland OR. The *Mycobacterium avium* genomic λZAPII library was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: *Mycobacterium avium* Genomic Library from the Division of AIDS, NIAID. Dr. Julia Inamine, Colorado State University, Fort Collins, CO, generously provided the genomic cosmid library of *Mycobacterium tuberculosis*. Electrocompetent MC1061/P3 *E.coli* were donated by Dr. Emily Platt, Oregon Heath Sciences University, Portland, OR. Purine analogs were obtained from Dr. Rich Miller, Burroughs Wellcome, Beckenham, England. Plasmid and cosmid vectors used included pBluescript (Strategene, LaJolla, CA), pBAce (Craig S.P.III et al., 1988) and pX63 (NEO and HYG), kindly provided by Stephen Beverley (Harvard Medical School). All restriction enzymes, DNA polymerases, ligases and calf intestinal phosphatase were purchased from either Gibco, BRL Life Technologies (Gaithersburg, MD), New England Biolabs (Beverly, MA), Strategene (La Jolla, CA), Epicentre (Madison, WI) or Borhringer Mannheim (Indianapolis, IN). Low-melt and ME agarose was purchased from USB (Cleveland, OH). Ready-to-go random prime labeling beads were from Pharmacia LKB Biotechnologies (Milwaukee, WI). Radiolabels [α-

^{32} P]dCTP (3000μCi/mmol), [γ-^{32} P]dATP (6000μCi/mmol), [α-^{35} S]dATP (1000-1500 Ci/mmol), and [α-^{35}S]dCTP (1000-1500 Ci/mmol) were all obtained from DuPont New England Nuclear (Boston, MA). Oligonucleotides were generated by DNAgency (Malvern, PA). Cocalico Biologicals (Reamstown,PA) produced the antibodies to XPRT. Vitamin Assay quality casamino acids were bought from Difco (Detroit, MI). All other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture: A clone of the Sudanese 1S strain of *L. donovani*, DI700, was grown to late log phase at room temperature in roller bottles containing DMEL (Iovannisci and Ullman, 1983) and supplemented with 100 μM xanthine.

Isolates 724 and 101 of *M. avium* and the Erdman strain of *M. tuberculosis* were grown in Proskhauer-Beck media (Youmans,1979) under a controlled environment of 5% CO_2, 37° C and 75% humidity located in a P3 facility using the biohazard safety guidelines outlined by the CDC.

E. coli strains XL1-blue (Bullock et al., 1987), JM109 (Yannish-Perron et al., 1985) and DH5α (Hanahan 1983) were grown under standard conditions in Luria-Bertani media (Sambrook et al., 1989). Other *E. coli* strains, Sϕ606 (*Δpro-gpt-lac, thi, hpt*) and Sϕ609 (*Δpro-gpt-lac, thi, hpt, pup, purHJ*)(Jochimsen et al., 1975) were cultured in low phosphate minimal media supplemented with appropriate purine sources as described previously (Craig S.P. III et al., 1991).

Nucleic acid isolation and analysis: Genomic or plasmic DNA from eiher *L. donovani* or *E. coli* were isolated by conventional alkaline lysis methods (Sambrook et al, 1989). Genomic mycobacterial DNA was extracted from cultures by a modification of a method by Folqueira et al.(1993). Addition of 1% glycine, final concentration, preceded lysis by one day. Following centrifugation, pelleted cells were resuspended in 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1% SDS and 0.2 N NaOH containing 100 µg/ml Proteinase K and incubated at 55° C for three hours. No noticeable lysis had occurred so Proteinase K was heat inactivated at 95° C for 15 min and Nonidet P-40 and Tween 20 were added to final concentrations of 0.45% and lysozyme at 10 mg/ml (essentially a modification of Lysis was allowed to occur overnight at 37° C and the genomic DNA was precipitated with a half volume of 3.0 M potassium acetate, pH 5.0. Centrifugation in a table top centrifuge (< 2500 rpms, 10 min) pelleted the genomic DNA, high molecular weight RNA, and potassium/SDS/protein/ membrane complexes. The pellet was resuspended in TE and incubated at 37° C in the presence of 10 µg/ml RNAse for 15 min followed by phenol/chloroform extraction (Sambrook et al., 1989).

Southern analysis was essentially performed as described (Sambrook et al., 1989).

Isolation of a M. avium hgprt fragment by the polymerase chain reaction: To generate a DNA probe for the isolation of the *M. avium* and *M. tubersulosis hgprt* a fragment of the *M. avium* gene was amplified from genomic DNA exactly as described previously (Allen and Ullman, 1993).

DNA sequence analysis: *L. donovani* sequence was determined by automated analysis completed by the core facility at Oregon Health Sciences University (Roger Cone Lab). Due to technical difficulties arising from a high G-C content, mycobacterial DNA was manually sequenced using a modification of the dideoxy-chain termination method of Sanger et al. (1977). DNA was heat denatured at 96° C for two minutes and placed on ice for an additional two minutes prior to alkali denaturation at 42° C (instead of 37° C) for five minutes. The tubes were immediately placed on ice and the DNA was quickly neutralized and ethanol precipitated. After washing, drying and resuspending the DNA in the usual fashion, the sequencing oligonucleotides were added. Annealment included a two minute incubation at 96° C followed by 10 minutes at 65° C and immediate placement on ice. The 7-deaza-dGTP reactions were immediately initiated to minimize creation of hairpins and prevent re-annealing of the DNA. The reaction included both ^{35}S-dCTP and ^{35} S-dATP allowing a higher incorporation of radiolabel which improved the readability of the resulting autoradiographs. The temperature of the extension reaction was adjusted from 37° C to 42° C to reduce

growth indicated the presence of a functional copy of *xprt*.

To determine the purine specificity of the mycobacterial phosphotransferases, the several coding regions that were possible were individually subcloned into the pBAce vector utilizing the same PCR-based strategy, transformed into Sϕ609 *E. coli* and plated as above except the experiment included testing for growth on adenine alone (negative control), or 150 μM guanosine (positive control), hypoxanthine, guanine or xanthine.

Overproduction of recombinant protein: Using polymerase chain reaction, the *xprt* and *hgprt* genes from *L. donovani* and the mycobacterium *M. avium* and *M. tuberculosis* respectively were amplified using specific 5' sense and 3' antisense oligonucleotides containing introduced in-frame restriction site overhangs, *NdeI* and *PstI* respectively. Subsequently each was subcloned into the 5' *NdeI* and 3' *PstI* sites of the pBAce vector (Craig S.P.III et al., 1988). Plasmid DNA isolated from the transformants was resequenced to verify accuracy. Sϕ609 *E. coli* transformed with pBAce constructs containing the respective phosphoribosyltransferase genes were grown in low phosphate induction media (LPI). LPI contained the complementary purine, either xanthine, guanine or hypoxanthine, which allowed overproduction of recombinant protein and ultimately ensured survival.

Purification of recombinant protein: One liter Sϕ609 *E. coli* cultures grown overnight were centrifuged at 3000 x g for 10 minutes and washed once in 250 ml TMD (20 mM Tris, 5 mM $MgCl_2$, 1 mM DTT (dithiotreitol), pH 7.4). The pellet was resuspended in 20 ml TMD and the cells were lysed under 10,000 p.s.i. in a french press. Lysate was clarified by centrifugation at 45,000 x g for 30 minutes (4° C). The supernatant was brought to 25% saturation with ammonium sulfate and incubated on ice for 1 hour the centrifuged at 45,000 x g for 45 minutes (4° C). The mycobacterial pellets contained > 90% HGPRT while the *L. donovani* pellet was small and consisted essentially of Sϕ609 *E. coli* proteins. Therefore, the *L. donovani* supernatant was brought to 70% ammonium sulfate saturation and incubated on ice for at least one hour. Recentrifugation, as above, pelleted the recombinant *L. donovani* XPRT.

Initially, the *M. avium* and *M. tuberculosis* HGPRTs were purified using a one step GTP-agarose affinity chromatography followed by elution with 100 µM PRPP (Allen et al., 1995a). Since poor yields were achieved, this method was only used infrequently. XPRT was partially resolved by CM-cellulose and DEAE chromatography. Both HGPRT's and XPRT were purified further to apparent homogeneity by gel permeation chromatography on a Sephadex G-100 column (100 x 2.7 cm).

Enzyme Assays: HGPRT and XPRT activities were assayed in 100 mM Tris, pH 7.4 containing 5 mM $MgCl_2$ using either radiometric (Iovannisci et al., 1984) or spectrophotometric (Tuttle and Krenitsky, 1979) methods.

Protein Quantitation: Protein concentrations were determined by the method of Bradford (1976).

Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed under reducing conditions in 15% acrylamide according to the method of Laemmli (1970).

Isoelectric focusing gel electrophoresis: Precast thin-layer agarose gel IEF was completed as described by the manufacturer (Isolab, Inc.; Akron, OH). The separatory pH range was 3-10 using a 0.5 M acetic acid anolyte and a 0.5 M NaOH catholyte. The power schedule consisted of stepwise increases as follows: 5W/5min., 10W/20min., 20W/5min. with the final voltage between 1100-1200. IEF protein standards (3.5-9.3) obtained from Sigma Chem. Co. (St. Louis, MO) were run to enable the determine the pI of both *M. avium* and *M. tuberculosis* HGPRT.

Purine Analog Screens Against M. tuberculosis and M. avium: Twenty-two purine analogs from Dr. Rich Miller, Burroughs Wellcome (Beckenham, UK) and seven others purchased from Sigma Chemical Co. (St. Louis MO, USA) (listed in the appendix on Table 1) were tested for growth inhibition against *M.*

avium strain #101 and *M. tuberculosis* Erdman strain. The analogs were dissolved in 0.05 N NaOH to a final concentration of 3 mg/ml (approximately 20 mM for each). One ml cultures containing 40 μl of added drug (approximately 800 μM final conc.) and either 1.15×10^6 colony forming units (CFU) of *M. avium* or 2.5×10^5 CFU of *M. tuberculosis* were incubated for one month as described above in this section under cell culture. Drugs shown to be effective were rescreened under similar conditions containing six individual dilutions (A-F) of drug varying from approximately 400 μM - 10 μM (0.06, 0.03, 0.015, 0.0075, 0.00375, 0.001875 mg/ml final concentration). Growth was observed for one month and a 100 μl aliquot of each culture visibly negative for growth was spread on 10 cm diameter plates containing 15 mls solid Middlebrook 7H11 agar media (Difco, Detroit, MI) to determine whether the effective analogs were inhibitory or bacterialcidal.

Crystallization protocol: Crystal Screens I and II (Jancarik and Kim, 1991, Hampton Research, Laguna CA, USA) were completed by the vapor diffusion hanging drop method. Each well contained 0.75 ml mother liquor. *M. tuberculosis* rHGPRT (2 μl of 10 mg/ml) was mixed on a coverslip with 2.4 μl of precipitating solution. The coverslip was then inverted and sealed to the well using high pressure vacuum grease. Additional setups of promising conditions were carried out in sitting drop wells containing 20 μl mother liquor and 20 μl of

10 mg/ml *M. tuberculosis* rHGPRT surrounded by 0.75 ml mother liquor.

RESULTS

A. *Leishmania donovani.*

Isolation and molecular characterization of the *L. donovani xprt*: The *L. donovani xprt* was isolated from a directionally cloned cDNA library (gift of Dr. Ken Stuart, Seattle Biomedical Research Institute, Seattle, WA) by complementation in Sϕ609 *E. coli* grown on xanthine supplemented LPI plates. Nucleotide sequence analysis of several clones indicated that all represented the same phophoribosyltransferase. A single clone was completely sequenced by automated analysis. A 50% homology was found between the deduced coding regions of the *L. donovani hgprt* and the putative *xprt* (Figure 14). To validate the cDNA sequence, a genomic clone was isolated from a *L. donovani* cosmid library (Hansen et al., 1992) by hybridization with the cDNA clone. The *xprt* cosmid clone was partially restriction mapped utilizing Southern analysis (Southern, 1975) and a 4.5 kb *EcoRI* fragment containing both the *L. donovani hgprt* and *xprt* (Figure 15) was subcloned into KS^+ pBluescript (with the help of Dr. Armando Jardim in the Ullman lab). The clone has been sequenced in its entirety and shows an orientation of 5' *hgprt* to *xprt* 3' (Figure 16). An *xprt* open reading frame of 723 bp encoding a 241 amino acid polypeptide was calculated to have a molecular mass of 27,023 Daltons (Figure 17) and a pI of 7.4. The *xprt* open reading frame was preceded by in-frame termination codons at

Figure 14. Alignment of XPRT and HGPRT from *L. donovani*.

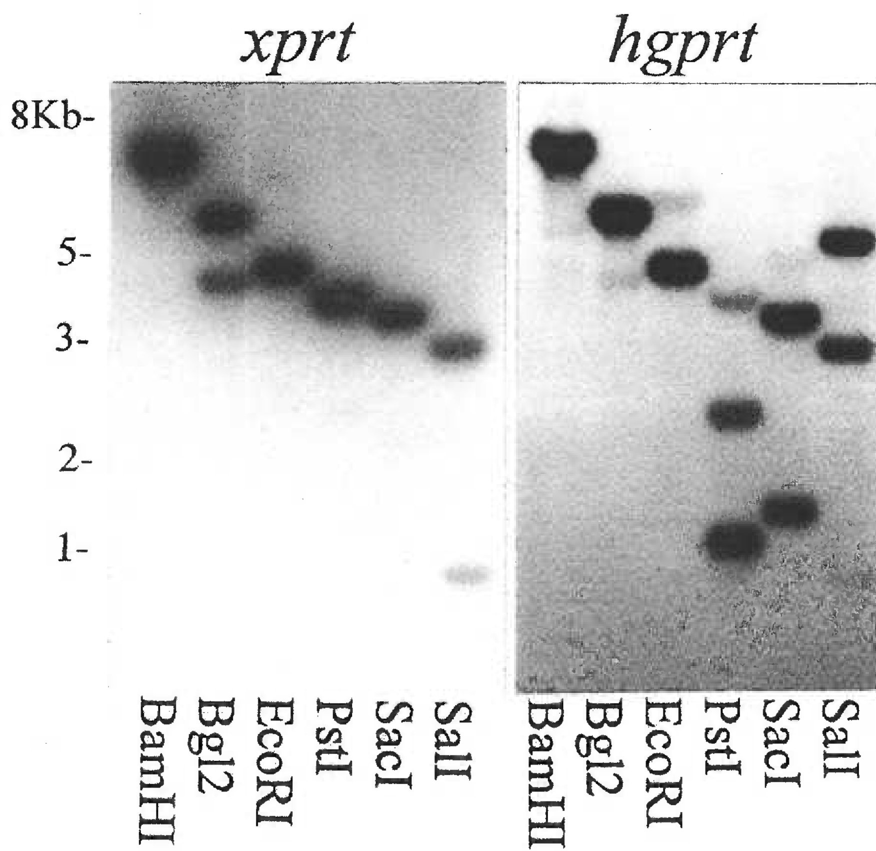
Alignment of the predicted amino acid sequence of *L. donovani* XPRT with HGPRT. A 26% identity is indicated by amino acid designations in pink while a 50% homology includes the those in light blue and green. Note that XPRT also contains a -COOH terminal degenerate glycosomal targeting signal lettered in red.

Alignment of XPRT and HGPRT from *L. donovani*

```
(X)   MLPTHSCKGFVDAQGRVFVDGREYPMASGIVATEDVIQTNIKAMAHTI[illegible]KDYKSLSHRDARLSPST
(HG)  M-------------------------SNSAKSPSGPVGDEGRRNYPM[illegible]AHTLVTQEQVWAATAKC

(X)   AETAEAAEA[illegible]EAPISYDNPLIIISVLKGSYIFT[illegible]D[illegible][illegible]R[illegible]L[illegible]DCGLPHVVDFVRLASYNSGTKSTGQ
(HG)  AKKIAEDYR[illegible]FKLTT-DNPLYLLCVLKGSFIFT[illegible]D[illegible][illegible]RFL[illegible]DEGVPVKVEFICASSYGTGVETSGQ

(X)   ISMLAGLRFENLRGKHVLIVEDVCDSGRTLRFLRDYIMEKFQPKSIKTLVMVNKEQAARKVDFDPE
(HG)  VRMLLDVR-DSVENRHILIVEDIVDSAITLQYLMRFMLAKK-PASLKTVVLLDKPSG-RKVEVLVD

(X)   YFCLAGPNKYIVGYGFEVNDRYRDLRHILILRDGEATRYPAKL
(HG)  YPVITIPHAFVIGYGMDYAESYRELRDICVLKKEYYEKPESKV
```

Figure 15. Southern analysis of a *L. donovani* genomic cosmid clone.

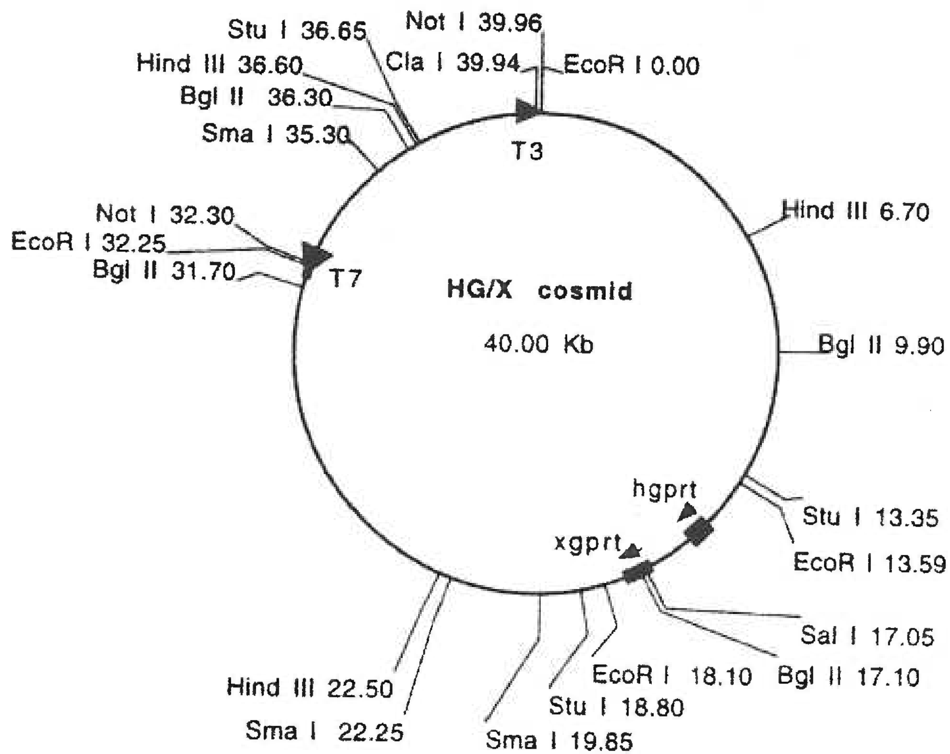
A genomic cosmid clone containing the *xprt* gene was digested individually with the restriction enzymes *BamHI, BglII, EcoRI, PstI, SacI* and *SalI* and analyzed by Southern hybridization. Blots were probed individually with randomly labeled *xprt* or *hgprt* genes.



xprt
hgprt
8Kb-
5-
3-
2-
1-
BamHI
Bgl2
EcoRI
PstI
SacI
SalI
BamHI
Bgl2
EcoRI
PstI
SacI
SalI

Figure 16. Cosmid map of clone containing xprt.

Partial restriction map of the cosmid clone generated using T_7, T_3, *xprt* and *hgprt* probes.



Not I 39.96
Stu I 36.65
Cla I 39.94
EcoR I 0.00
Hind III 36.60
Bgl II 36.30
Sma I 35.30
T3
Hind III 6.70
Not I 32.30
EcoR I 32.25
Bgl II 31.70
T7
HG/X cosmid
40.00 Kb
Bgl II 9.90
hgprt
Stu I 13.35
EcoR I 13.59
xgprt
Sal I 17.05
EcoR I 18.10
Bgl II 17.10
Stu I 18.80
Hind III 22.50
Sma I 22.25
Sma I 19.85

Figure 17. Nucleotide sequence of the *L. donovani xprt* and deduced amino acid sequence.

Shown are 1080 bp of the 4.5 kb *EcoRI* fragment *xprt* clone, sequenced in both directions. The amino-acid sequence of the 723 bp open reading frame was deduced. In-frame stop codons at positions -15, -135 and -144 are shown in yellow. The splice leader junction is marked by a red AG.

Leishmania donovani xprt

```
AATCAACACGCATGAACACCTGCACACATTCTCGGGCACCGATCCGCCCATCAGGAAGAG    60
GCCCCCTCCCCTCCCCCGCTCCAAGGGAAAGACGCATAAGAGAAATAAAGGAGTCTTGCT   120
CACTCTGCGTTCACCACACTCGCAGGCAACCACCGGCGGCGCACCGCACCGCACCGCA   180
CGCTCACGCTCTGGCTGCACAGCACACGCGCACGCAACGGAGCGATAGATAAATACATCA   240
ATGCTACCAACCCACAGTTGTAAAGGTTTCGTGGATGCCCAGGGCAGGGTCTTCGTGGAT   300
 M  L  P  T  H  S  C  K  G  F  V  D  A  Q  G  R  V  F  V  D
GGCCGCGAGTACCCCATGGCGTCTGGCATTGTCGCCACGGAAGACGTAATCCAGACGAAC   360
 G  R  E  Y  P  M  A  S  G  I  V  A  T  E  D  V  I  Q  T  N
ATCAAGGCCATGGCGCACACAATTGCGAAGGACTACAAGTCGCTCAGCCACCGCGACGCT   420
 I  K  A  M  A  H  T  I  A  K  D  Y  K  S  L  S  H  R  D  A
CGTCTGTCACCCAGCACGGCGGAGACCGCAGAGGCGGCAGAGGCGGCGGAGGCGCCGATC   480
 R  L  S  P  S  T  A  E  T  A  E  A  A  E  A  A  E  A  P  I
AGCTACGACAACCCGCTCATCATCATCTCCGTGCTCAAGGGCAGCTACATCTTCACATCC   540
 S  Y  D  N  P  L  I  I  I  S  V  L  K  G  S  Y  I  F  T  S
GACTTCATCCGCTACCTCGGCGACTGCGGCCTGCCGCACGTTGTCGACTTTGTGCGGTTG   600
 D  F  I  R  Y  L  G  D  C  G  L  P  H  V  V  D  F  V  R  L
GCCTCGTACAACTCGGGTACAAAGAGCACCGGCCAGATCTCGATGCTGGCGGGTCTCAGA   660
 A  S  Y  N  S  G  T  K  S  T  G  Q  I  S  M  L  A  G  L  R
TTCGAGAATCTACGCGGCAAGCACGTACTGATCGTCGAGGATGTGTGCGATTCTGGGCGC   720
 F  E  N  L  R  G  K  H  V  L  I  V  E  D  V  C  D  S  G  R
ACGCTGCGCTTCCTGCGCGATTACATCATGGAGAAGTTCCAGCCCAAGAGCATCAAGACG   780
 T  L  R  F  L  R  D  Y  I  M  E  K  F  Q  P  K  S  I  K  T
CTCGTGATGGTGAACAAAGAGCAGGCGGCCCGCAAGGTGGACTTCGATCCGGAGTACTTC   840
 L  V  M  V  N  K  E  Q  A  A  R  K  V  D  F  D  P  E  Y  F
TGCCTTGCCGGCCCAAACAAGTACATTGTCGGATACGGGTTCGAGGTGAACGATCGCTAC   900
 C  L  A  G  P  N  K  Y  I  V  G  Y  G  F  E  V  N  D  R  Y
CGTGACTTGCGTCACATCCTCATCCTGCGGGACGGGGAGGCCACCCGTTACCCTGCCAAG   960
 R  D  L  R  H  I  L  I  L  R  D  G  E  A  T  R  Y  P  A  K
CTCTGAGCTCGACGTCACACCACCGGAGTGGAGGGAAATGTGGAGGCGGCTGAGTGTGCC  1020
 L STOP
GGAGTAAGAGAAGTAAGGGAGCCTGCGGAGAAGACGCTTGTGCACGTGTGTATGCCCCCA  1080
```

positions -15, -135 and -144 upstream of the predicted start codon. Southern analysis of *L. donovani* DI700 genomic DNA digested individually with *ClaI, KpnI, NdeI, PstI, SacI, SalI* and *XhoI* suggested that *xprt* (Figure 18) like *hgprt* (Allen et al., 1995a) is a single copy gene. Sequence analysis of a 5' *xprt* fragment generated by PCR from *L. donovani* DI700 cDNA using a nondegenerate sense primer to the mini-exon and an antisense sequencing primer to the middle of the gene determined the splice leader site as shown in red in Figure 17.

Overexpression of the *L. donovani xprt* in *E. coli:* The *L. donovani xprt* was generated by PCR, ligated into the pBACe bacterial expression vector (Craig III et al., 1991) and transformed into Sϕ609 *E. coli* for complementation studies and overproduction of recombinant XPRT protein. Complementation of pBAce-*xprt* and pBAce expressing Sϕ609 *E. coli* on LPI-agarose plates containing either 150 mM guanosine (positive control), hypoxanthine, guanine, xanthine, or no additional purine (negative control) confirmed that the *L. donovani xprt* complemented the Sϕ609 lesion and supported growth only on media supplemented with xanthine or on a positive control plate containing guanosine (Shown in Figure 19). Prolonged incubation on guanine or hypoxanthine containing plates resulted in the appearance of small colonies, indicating a limited capacity of the *L. donovani xprt* to utilize guanine and hypoxanthine as substrates (data not shown).

Figure 18. Southern blot analysis of the *L. donovani* DI700 *xprt* locus.

Genomic DNA isolated from DI700 *L. donovani* (5 μg) was digested to completion with the indicated restriction endonucleases and subjected to Southern blot analysis utilizing the cDNA *xprt* as a hybridization probe.

Genomic DNA

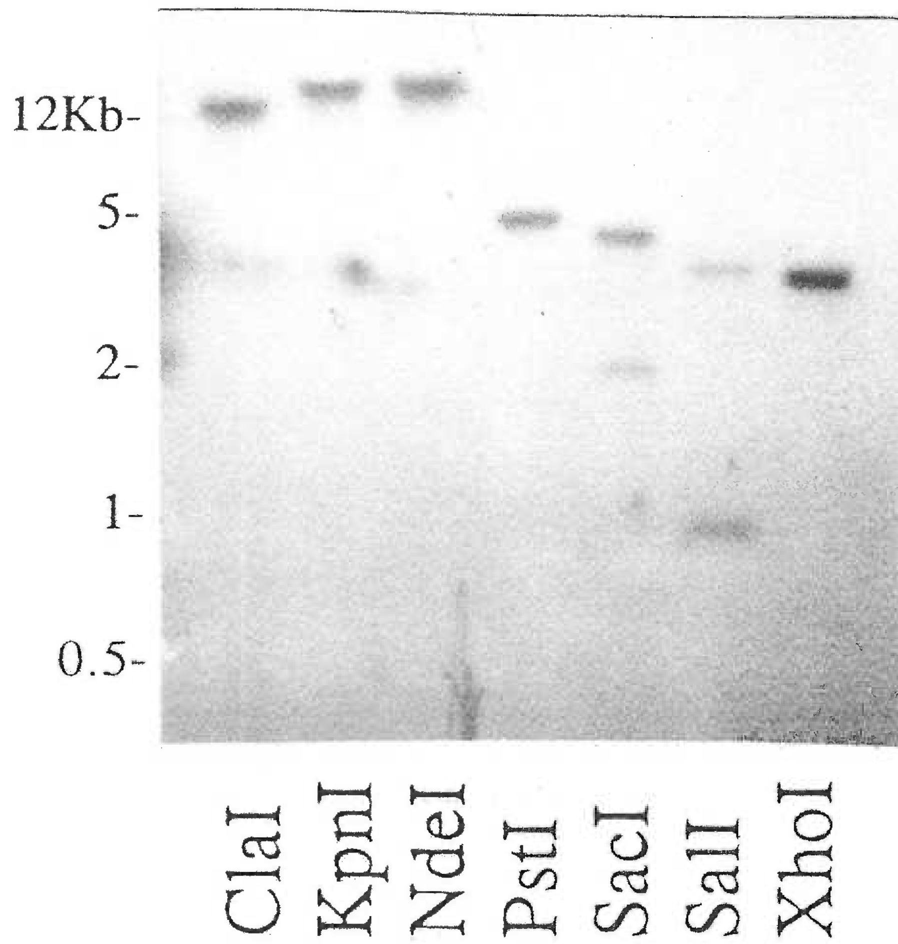
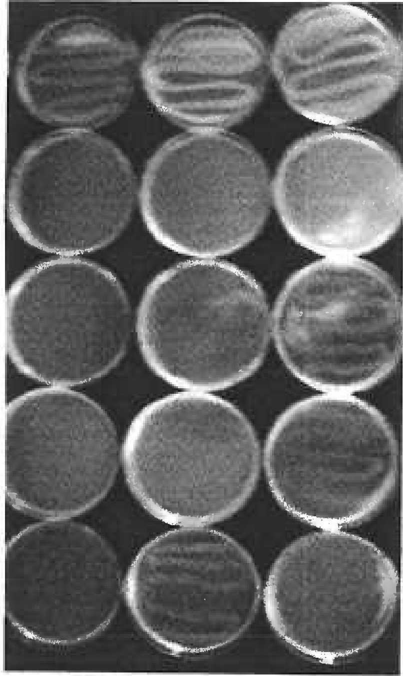


Figure 19. Complementation of bacterial *g(x)pt* mutation.

The pBAce and pBAce-*xprt* plasmids were transformed into Sϕ609 *E. coli* and plated on LPI-agarose containing either adenine plus guanosine as a positive control, no purine as a negative control, hypoxanthine, guanine or xanthine at a concentration of 150 μM as indicated.



pBAce only	XPRT	HGPRT	
+	+	+	Adenine\Guanosine
−	−	−	No Purine
−	−	+	Hypoxanthine
−	−	+	Guanine
−	+	−	Xanthine

Sϕ609 *E. coli*

When pBAce-*xprt* Sϕ609 *E. coli* are induced in LPI-liquid media, the recombinant XPRT is the predominant protein produced. As shown by 15% PAGE (Figure 20), a band at approximately 30 kDa was observed in extracts of pBAce-*xprt* Sϕ609 *E. coli* but not in Sϕ609 *E. coli* containing only the vector pBAce grown in guanosine.

Purification and characterization of the recombinant XPRT protein: The recombinant XPRT produced in Sϕ609 *E. coli* was purified by column chromatography. The protein in 20 mM Tris pH 7.4 containing 5 mM $MgCl_2$ and 1mM DTT was passed over a CM-cellulose column followed by DEAE. The eluate was further resolved to apparent homogeneity by two passes over a 300 ml G100-120 sephadex gel-filtration sizing column. Purified XPRT was used to immunize rabbits and the antibody produced is currently in the process of being characterized.

Initial kinetic studies were completed by Dr. Armando Jardim (Ullman lab) and me (Figure 21). As expected, the XPRT catalyzed the phosphoribosylation of xanthine and Hanes plots were used to calculate K_m and k_{cat} values of 9.5 μM and 2.5 s^{-1} for xanthine. Hypoxanthine and guanine were also found to be substrates, albeit poor ones, with K_m and k_{cat} values of 37 μM and 0.026 s^{-1} for hypoxanthine.

Figure 20. Overproduction and purification of recombinant XPRT.

Protein was produced in *E. coli* Sϕ609 cells genetically deficient in *g(x)prt* and *hpt,* transformed with leishmanial *xprt* in the pBAce vector. Molecular mass standards are shown on the left of the SDS-PAGE. XPRT is indicated and has been purified as described in Materials and Methods. Total protein from *E. coli* Sϕ609-*xprt* extract is also shown.

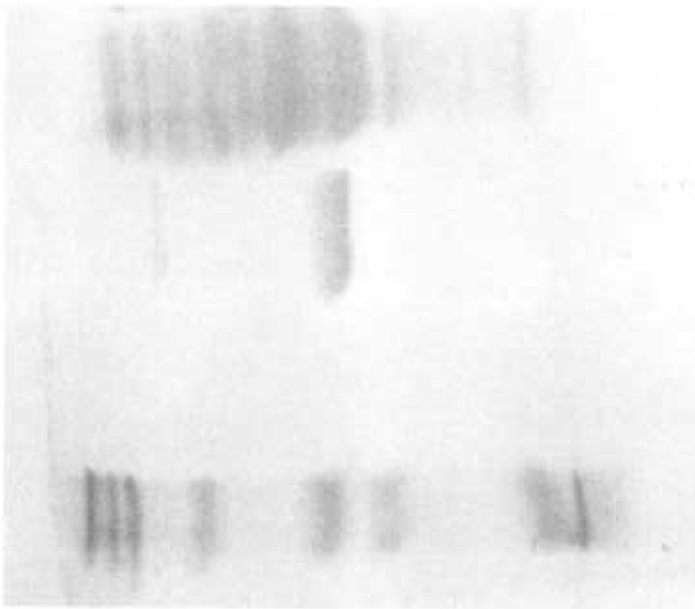
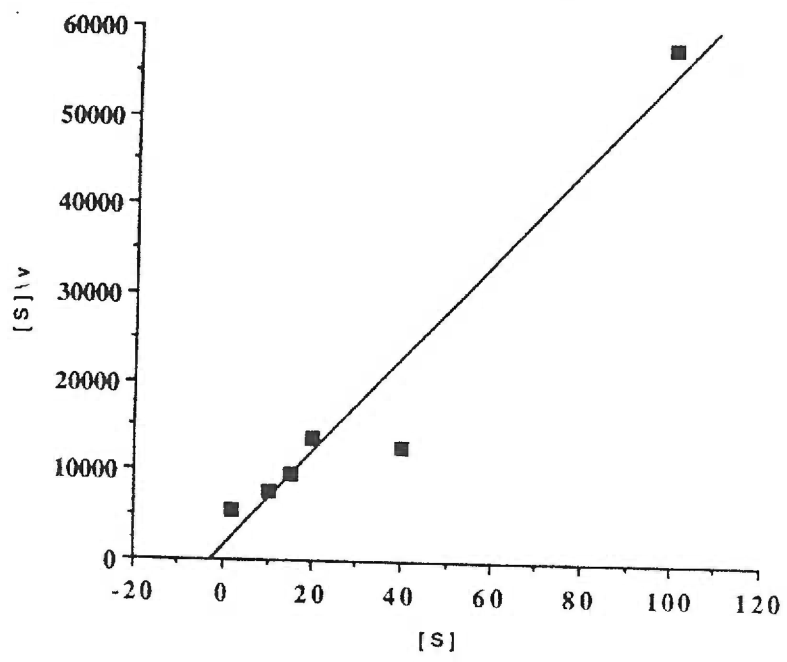

Crude lysate
L. donovani XPRT
STD

Figure 21. Hanes analysis of recombinant *L. donovani* XPRT.

L. donovani XPRT was assayed spectrophotometrically at 25° C with xanthine (data shown), hypoxanthine, guanine and adenine. K_ms of 9.5 and 37 μM and k_{cat} values of 2.5 and 0.026 s^{-1} were determined for xanthine and hypoxanthine (data not shown) respectively. Initial guanine kinetics suggest that it is a poor substrate for XPRT. No adenine phosphoribosylation could be detected.


Xanthine
60000
50000
40000
30000
20000
10000
0
[S]\v
-20
0
20
40
60
80
100
120
[S]

Data collection for guanine is incomplete at this time. A further complication is that when a new sizing column was poured with a different lot of G100-120 it was not able to completely resolve XPRT to visual homogeneity. Consequently, further characterization is necessary and ongoing.

B. *Mycobacterium avium* and *Mycobacterium tuberculosis.*

Isolation and molecular characterization of *hgprt* from *M. avium* and *M. tuberculosis*: Degenerate primers created from conserved regions among other HGPRT proteins (Allen and Ullman, 1993) were used to amplify a 174 bp fragment of the *M. avium hgprt* gene from genomic DNA using PCR. Genomic southern analysis of both *M. avium* and *M. tuberculosis* using the 174 bp *M. avium hgprt* gene fragment as a probe indicated the presence of a *hgprt* gene in each organism (Figure 22). Therefore, the fragment was used to isolate, by hybridization, full length *hgprt* from both *M. avium* and *M. tuberculosis*. The *M. avium* clone containing *hgprt* was isolated from an NIH λZAPII genomic library provided by the AIDS Research and Reference Reagent Program. The *M. tuberculosis hgprt* was cloned from a genomic cosmid library obtained as a gift from Dr. Julia Inamine, Colorado State University, Fort Collins CO. Southern analysis of the *M. tuberculosis* clone restriction digested with *BamHI, ClaI, EcoRI, HindIII, NotI, PstI and SacI* was completed (Figure 23). A 4 kb *NotI* fragment containing the *hgprt* was subcloned into KS^+ pBS and the *M. tuberculosis* as well as the *M. avium hgprt* from the λZAPII clone were sequenced in their entirety by a modified 7-deaza dideoxy chain termination sequencing method (Sanger, 1977) described in the methods section, to increase read through of G-C rich DNA. Oligonucleotide primers used for sequencing were derived

Figure 22. Southern blot analysis of *M. avium* and *M. tuberculosis* chromosomal DNA.

DNA isolated from *M. avium* isolate 724 and *M. tuberculosis* Erdman strain were digested with the designated restriction enzymes, blotted to Nytran filters, and probed with the 174 bp *M. avium hgprt* fragment.

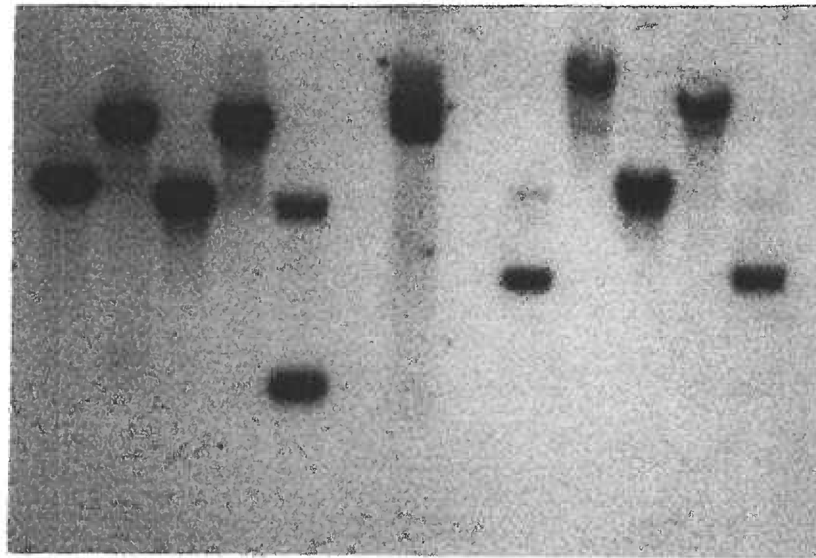
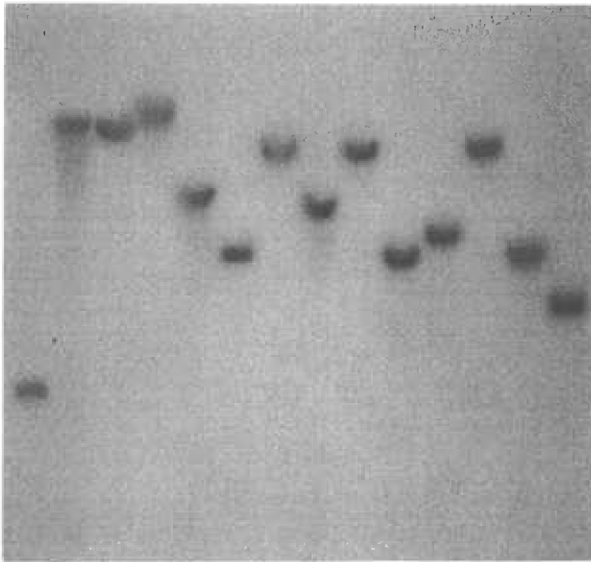
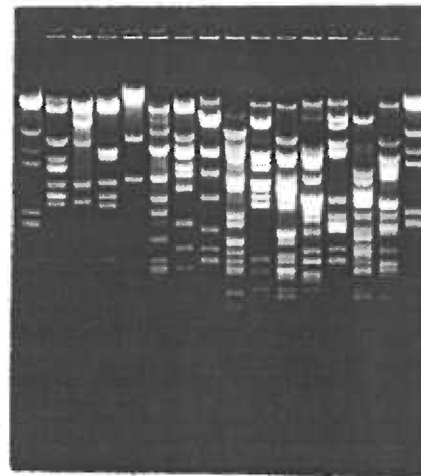

M. avium
M. tuberculosis
14.7 kb -
6.9 -
4.7 -
1.9 -
0.9 -
0.25 -
BamHI
EcoRI
PstI
SacI
SalI
BamHI
EcoRI
PstI
SacI
SalI

Figure 23. Southern analysis of the *M. tuberculosis hgprt* cosmid clone.

A *M. tuberculosis* cosmid clone was isolated by screening with the 174 bp *M. avium hgprt* fragment. Shown here is a Southern blot of the clone digested with the restriction enzymes listed. The *NotI* fragment was subcloned into pBS and used to sequence the entire *M. tuberculosis hgprt*.

Southern Analysis of *M. tuberculosis hgprt* clone





from the known sequence of the *M. avium hgprt* 174 bp PCR fragment. Possible primer sequences were analyzed by the computer program Oligo (shareware from NIH) to determine interfering hairpin structures. As is the case with any CG rich genome, producing functional oligo primers can be a problem. Many iterations were usually necessary to determine a suitable sequence. Loop Tm's of greater than 90°C were common. Another recurring problem was that of multiple primer sites. As sequence was collected it was entered into the IBI Putstell program and an alignment was made between the putative oligo and all DNA sequence so far determined. If it was computed to be thermodynamically feasible, and the 3' end was compatible for possible multiple priming, another oligonucleotide sequence was choosen. In cases where it could not be known *a priori*, but multiple priming occurred anyway, new primers were again designed and tested until one was discovered that worked. The completed sequence of both genes is shown in Figures 24 and 25.

Overexpression of the *M. avium* and *M. tuberculosis hgprt* in *E. coli:* A search for open reading frames in the *M. avium* sequence revealed several posibilities given that the mycobacteria start codon may be ATG or GTG. Either is used in equal frequency. And on rare occasion TTG may also be used. The Shine-Dalgarno sequence is not well conserved and did not help eliminate any possibilities. The boxes -10 and -35 can be in front of or after the start site and

Figure 24. Nucleotide sequence of the *M. avium hgprt* and deduced amino acid sequence.

Shown is the *M. avium hgprt* λZAPII genomic clone isolated by hybridization with the 174 bp PCR *hgprt* fragment, sequenced in both directions. The amino acid sequence of the putative 609 nucleotide open reading frame was deduced. The assumed initiating methionine codon is a GTG shown in green. Also indicated in light blue are other possible start sites that could theoretically have been initiating codons in *M. avium*.

Mycobacterium avium hgprt

```
GCAGCCCGGCGAGCGGCTGTTCGGGGCGCCGCGCGGACATGTTGACGCTGTGGCGCGAGC
CGGTCCGTTAACCGCAATGCCGGAGCCGCCGTTGGTTATTCGCCGGCCGGCTGGCACGCT
GTGGGCGTGGCCCAGATCTCCTCGGCGATCACCCCCGCCCAGCCCGCGGAGCTGTACCCC     60
fM G  V  A  Q  I  S  S  A  I  T  P  A  Q  P  A  E  L  Y  P
GGGGATATCAAGTCTGTCCTGCTGACGGCGGAGCAAATTCAGGCCCGCATCGCCGAACTC    120
 G  D  I  K  S  V  L  L  T  A  E  Q  I  Q  A  R  I  A  E  L
GGCCGCGAAATCGGGGACAACTACCGCGATGCCGCGGCCGAGACCGGACAGGACCTGCTG    180
 G  R  E  I  G  D  N  Y  R  D  A  A  A  E  T  G  Q  D  L  L
CTGATCACCGTGCTCAAGGGCGCTGTGATCTTTGTCACCGACCTGGCCCGGGCGATTCCG    240
 L  I  T  V  L  K  G  A  V  I  F  V  T  D  L  A  R  A  I  P
CTGCCCACCCAGTTCGAATTCATGGCCGTCAGCTCCTACGGGTCGTCGACGTCGTCGTCG    300
 L  P  T  Q  F  E  F  M  A  V  S  S  Y  G  S  S  T  S  S  S
GGCGTGGTGCGCATCCTCAAGGACCTGGACCGTGACATCCAGGGCCGCGACGTGCTGATC    360
 G  V  V  R  I  L  K  D  L  D  R  D  I  Q  G  R  D  V  L  I
GTCGAGGACGTCGTGGACTCGGGGCTGACGCTGTCCTGGCTGCTGCGCAACCTGAGCACC    420
 V  E  D  V  V  D  S  G  L  T  L  S  W  L  L  R  N  L  S  T
CGTCATCCTCGGTCGCTGCGGGTGTGCACGCTGTTGCGCAAGCCCGACGCCAGGGGCGCC    480
 R  H  P  R  S  L  R  V  C  T  L  L  R  K  P  D  A  R  G  A
CACGTCGACATCGCCTACGTGGGGTTCGACATCCCCAACGACTTCGTGGTGGGATACGGC    540
 H  V  D  I  A  Y  V  G  F  D  I  P  N  D  F  V  V  G  Y  G
CTCGACTACGACGAGCGCTACCGCGACCTGTCCTACATCGGCACCCTGGACCCCAGGGTC    600
 L  D  Y  D  E  R  Y  R  D  L  S  Y  I  G  T  L  D  P  R  V
TACCAGCAGTAGCTCCCGCGCTCCCGCTCAGTCCAGCTCCCAGGACGCGGTGACGGTGAA    609
 Y  Q  Q  STOP
CTTCACCGTCTGCTGGCCCGGTTCCAGCGGCACCGCCATCGGCATCGCGCGCGGCGACGG
```

Figure 25. Nucleotide sequence of the *M. tuberculosis hgprt* and deduced amino acid sequence.

Shown is the *M. tuberculosis hgprt* λZAPII genomic clone isolated by hybridization with the 174 bp PCR *hgprt* fragment, sequenced in both directions. The amino acid sequence of the putative 606 nucleotide open reading frame was deduced. The assumed initiating methionine codon is a GTG shown in green. Also indicated in light blue are other in frame codons which could theoretically have been initiating codons in *M. tuberculosis.*

Mycobacterium tuberculosis hgprt

```
CGGTGGGTCCACGTTGCGCGGTCAGCGACTGGTCGCCGGGCGGCGCGACGGCGTACTTGTG
TTGCGGCGCGAGCCGTTTGACGCCCGCGTTGGTCGTTGGTCCCGCGGCGGCGTGGCACGCT
GTGCACGTGACCCAGAGCTCCTCGGCGATCACCCCCGGGCAGACGGCGGAGCTTTATCCG    60
fM  H  V  T  Q  S  S  S  A  I  T  P  G  Q  T  A  E  L  Y  P
GGGGACATCAAGTCGGTGCTGCTCACGGCCGAGCAGATTCAGGCCCGCATCGCCGAGCTC   120
 G  D  I  K  S  V  L  L  T  A  E  Q  I  Q  A  R  I  A  E  L
GGCGAGCAGATCGGCAACGACTACCGCGAGCTGTCCGCTACCACCGGCCAGGATCTGCTG   180
 G  E  Q  I  G  N  D  Y  R  E  L  S  A  T  T  G  Q  D  L  L
CTGATCACCGTGCTGAAGGGCGCGGTGCTCTTCGTCACCGACCTGGCGCGAGCGATTCCC   240
 L  I  T  V  L  K  G  A  V  L  F  V  T  D  L  A  R  A  I  P
GTGCCGACCCAGTTCGAGTTCATGGCGGTGAGTTCGTATGGGTCATCGACATCCTCGTCG   300
 V  P  T  Q  F  E  F  M  A  V  S  S  Y  G  S  S  T  S  S  S
GGCGTGGTGCGGATCCTCAAGGACCTCGACCGCGACATCCACGGCCGCGACGTGCTGATC   360
 G  V  V  R  I  L  K  D  L  D  R  D  I  H  G  R  D  V  L  I
GTCGAGGACGTCGTCGACTCCGGCCTTACGCTTTCGTGGTTGTCGCGGAACCTGACGAGC   420
 V  E  D  V  V  D  S  G  L  T  L  S  W  L  S  R  N  L  T  S
CGGAATCCGCGGTCATTGCGGGTGTGCACGCTGCTGCGCAAGCCCGATGCGGTGCACGCC   480
 R  N  P  R  S  L  R  V  C  T  L  L  R  K  P  D  A  V  H  A
AACGTCGAAATCGCGTACGTGGGTTTCGACATTCCCAAGACCTTCGTCGTGGGCTACGGC   540
 N  V  E  I  A  Y  V  G  F  D  I  P  K  T  F  V  V  G  Y  G
CTGGACTACGACGAACGCTACCGTGACCTGTCATACATCGGGACGCTGGACCCCAGGGTC   600
 L  D  Y  D  E  R  Y  R  D  L  S  Y  I  G  T  L  D  P  R  V
TATCAGTAGGCGGCTAGGTCAGTTCCCAGACCACCGTGACCGAGAAGCCCACCGTCTGCT   606
 Y  Q STOP
GGCCGGGTTCCAGCGGCACCGCGGATAGGCCGCCGGCCTAGCCCGGCGCCTCGTGCGTGG
```

also were no use in determining the correct open reading frame. Another concern was that these genes might code for proteins that are spliced as can occur in mycobacterial species. Translation in all three reading frames did not reaveal a conserved splice site sequence. Upon alignment of the two mycobacterial *hgprt* gene sequences it was noticed that at a certain point a higher degree of homology occurred (data not shown). This information, coupled with knowledge of the length of several disparately related *hgprt*s and their, albeit limited, homology to each other caused us to take the approach of subcloning several clones with different posible starts into the pBAce expression vector (Craig III, 1991). The *M. avium* and *M. tuberculosis xprt* genes had two sets of GTGs in common as well as other putative starts sites, however we did not want to assume that they would have identical 5' termini. The various clone start sites are shown in Figure 26. Complementation on hypoxanthine or guanine LPI-agarose plates of the pBAce-*xprt* A-E clones transformed into Sϕ609 *E. coli* resulted in growth of only clones A and B from both *M. avium* and *M. tuberculosis* (Figure 27). Clone B from both mycobacterium was choosen to represent the *hgprt* gene as further proof of the *in vivo* start site was not possible to provide from our laboratory. Presumably, 609 and 606 nucleotide open reading frames code for HGPRT proteins 24 kD and 23.9 kD respectively for *M. avium* and *M. tuberculosis* (see again Figures 24 and 25).

Figure 26. Alignment of *M. avium* and *M. tuberculosis* 5' upstream *hgprt* region.

Lack of any definitive start site indicators led to the use of the alignment of the two putative *hgprts* to determine all possible and reasonable translational start sites. The *M. avium hgprt* upstream sequence is shown in dark blue while that of *M. tuberculosis* is indicated in violet. Possible start sites are indicted by orange or green with green sites showing common possible start codons. Oligonucleotides were created to each feasible initiation site and the putative *hgprt*s were subcloned into pBAce vector utilizing a PCR-based approach.

The subclone used in the studies described in this thesis is depicted as starting at the probable start codon label A. Sequence within the genes is shown in sky blue and lilac. B is an in frame gtg codon and could theoretically be the initiating codon.

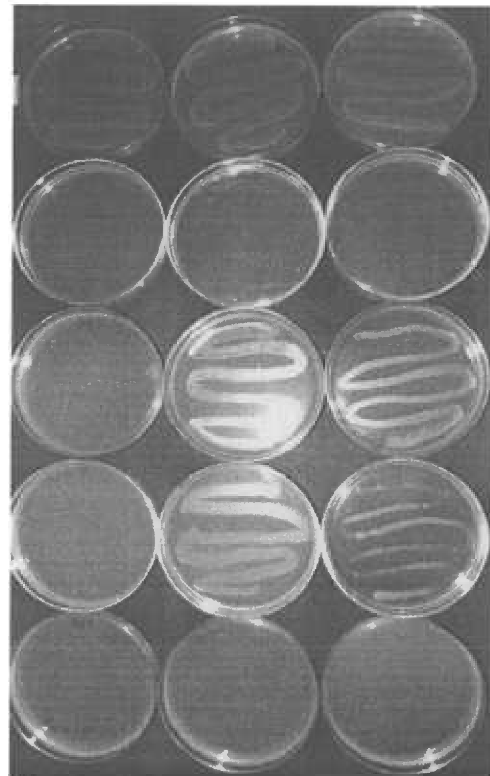
Mycobacterium 5' - *hgprt* comparison

```
M. avium            CGGACATGTTGACGCTGTGGCGCGAGCCGG
M. tuberculosis     CCGGGCGGCGCGACGGCGTACTTGTGTTGC

                    TCCGTTAACCGCAATGCCGGAGCCGCCGTT
                    GGCGCGAGCCGTTTGACGCCCGCGTTGGTC

                                           5'- A
                    GGTTATTCGCCGGCCGGCTGGCACGCTGTG
                    GTTGGTCCCGCGGCGGCGTGGCACGCTGTG

                       B ⇨
                    GGCGTGGCCCAGATCTCTTCGGCGATCACC
                    CACGTGACCCAGAGCTCCTCGGCGATCACC
```

Figure 27. Complementation of *hpt* and *gpt* mutations in Sϕ609 *E. coli* with *M. avium* and *M. tuberculosis hgprt*.

The pBAce and pBAce-*hgprt* plasmids were transformed into Sϕ609 *E. coli* and plated on LPI-agarose containing either adenine plus guanosine as a positive control, no purine as a negative control, hypoxanthine, guanine or xanthine at a concentration of 150 μM as indicated.

Sϕ609 *E. coli* Complementation



Guanosine

No Purine

Hypoxanthine

Guanine

Xanthine

pBAce only

M. t. HGPRT

M. a. HGPRT

Primary sequence alignment of various known HGPRTs: Alignment of the deduced primary sequence (Figure 28) of various HGPRTs reveals that these enzymes have relatively limited homology. Three short significant stretches are underlined in red and correspond to regions within the active site as determined by crystal structures solved by X-ray diffraction (Eads et al., 1994, Schumacher et al., 1996, Somoza et al., 1996). The conserved region closest to the amino terminus appears to surround the ribose phosphate moiety of the bound product. The phosphoribosylpyrophosphate (PRPP) binding motif is indicated by the center region in red while the carboxyl terminal area with high homology is involved in binding the purine ring. A comparison of identity seen in a variety of HGPRT enzymes indicates a diversity of primary structures with a low of 23% seen between *L. donovani* and *P. falciparum* and a high of 78% between the related organisms *L. donovani* and *C. fasciculata* (Figure 29).

Purification and characterization of the recombinant mycobacterial HGPRT protein: The recombinant *M. avium* and *M. tuberculosis* HGPRT enzymes were purified to apparent homogeneity by differential ammonium sulfate precipitation followed by molecular sizing on a G100-120 sephadex column as described in the methods. The substrate specificity of the purified *M. tuberculosis* HGPRT was evaluated. As expected the enzyme catalyzed the phosphoribosylation of hypoxanthine or guanine but not adenine or xanthine.

Figure 28. Multiple sequence alignment of various HGPRT's.

Alignments of the primary structures of the *Mycobacterium avium, Mycobacterium tuberculosis, Vibrio harveyi, Leishmania donovani, Trypanosoma brucei, Trypanosoma cruzi, Plasmodium falciparum, Crithidia fasciculata* and human HGPRT enzymes. An asterick (*) denotes complete identity while a period (.) allows for a conservative substitution.

CLUSTAL V multiple sequence alignment

```
M.avium         MGVAQISSAITPAQPA-----E------------LYPGDIKSVLLTAEQI
M.tuberculosis  MHVTQSSSAITPGQTA-----E------------LYPGDIKSVLLTAEQI
V.harveyi       MKHTVE--------------------------------------VMISEQEV
L.donovani      MSNSAKSPSGPVG--------DEG--------RRNYPMSAH-TLVTQEQV
T.brucei        MEPACK-----------------------------YDFATS-VLFTEAEL
T.cruzi         MP-----------------------------REYEFAEK-ILFTEEEI
P.falciparum    MPIPNNPGAGENAFDPVFVKDDDGYDLDSFMIPAHYKKYLTKVLVPNGVI
C.fasciculata   MSNAASPATSAAP-------------------VRHYPMSCR-TLATQEQI
HUMAN           MATRS-PGV-------VISDDEPGYDLDLFCIPNHYAEDLERVFIPHGLI
                *

M.avium         QARIAELGREIGDNYRDAAAETGQD-LLLITVLKGAVIFVTDLARAIP--
M.tuberculosis  QARIAELGEQIGNDYRELSATTGQD-LLLITVLKGAVLFVTDLARAIP--
V.harveyi       QERIRELGKQITERYQGSE--D----LVMVGLLRGSFVFMA-DLAR----
L.donovani      WAATAKCAKKIAEDYRSFKLTT-DNPLYLLCVLKGSFIF-TADLARF---
T.brucei        HTRMRGVAQRIADDYSNCNLKPLENPLVIVSVLKGSFVF-TADMVRI---
T.cruzi         RTRIMEVAKRIADDYKGKGLRPYVNPLVLISVLKGSFMF-TADLCRA---
P.falciparum    KNRIEKLAYDIKKVYNN-------EEFHILCLLKGSRGFFTALLKHLSRI
C.fasciculata   WSATAKCAKQIAEDYKQYNLSD-ENPLYLLCVLKGSFMF-TADLARF---
HUMAN           MDRTERLARDVMKEMGG-------HHIVALCVLKGGYKFFADLLDYIKAL
                                          .  . .*.*.  *    .

M.avium         -------LPTQF---EFMAVSSYGSSTSSSGVV-RILKDLDRDIQGRDVL
M.tuberculosis  -------VPTQF---EFMAVSSYGSSTSSSGVV-RILKDLDRDIHGRDVL
V.harveyi       ----AIELTHQV---DFMTASSYGNTMESSRDV-RILKDLDDDIKGKDVL
L.donovani      --LADEGVPVKV---EFICASSYGTGVETSGQV-RMLLDVRDSVENRHIL
T.brucei        --LGDFGVPTRV---EFLRASSYGHDTKSCGRV-DVKADGLCDIRGKHVL
T.cruzi         --LSDFNVPVRM---EFICVSSYGEGVTSSGQV-RMLLDTRHSIEGHHVL
P.falciparum    HNYSAVEMSKPLFGEHYVRVKSYCND-QSTGTLEIVS-EDLSCLKGKHVL
C.fasciculata   --LCDEGVPVRI---EFICASSYGTDVKTSGEV-RLLLDVRDPVENRHLL
HUMAN           NRNSDRSIPMTV---DFIRLKSYCND-QSTGDIKVIGGDDLSTLTGKNVL
                               .    .**     . .          . ..*

M.avium         IVEDVVDSGLTLSWLLRNLSTRHPRSLRVCTLLRKPDARGAHVDIAYVGF
M.tuberculosis  IVEDVVDSGLTLSWLSRNLTSRNPRSLRVCTLLRKPDAVHANVEIAYVGF
V.harveyi       IVEDIIDTGNTLNKIREILSLREPKSIAICTLLDKPSRREVEVPVDYVGF
L.donovani      IVEDIVDSAITLQYLMRFMLAKKPASLKTVVLLDKPSGRKVEVLVDYPVI
T.brucei        VLEDILDTALTLREVVDSLKKSEPASIKTLVAIDKPGGRKIPFTAEYVVA
T.cruzi         IVEDIVDTALTLNYLYHMYFTRRPASLKTVVLLDKREGRRVPFSADYVVA
P.falciparum    IVEDIIDTGKTLVKFCEYLKKFEIKTVAIACLFIKRTPLWNGFKADFVGF
C.fasciculata   IVEDIVDSAITLEYLKRFLNAKNPASLKTVVLLDKPSGRKVTLSVDYPVI
HUMAN           IVEDIIDTGKTMQTLLSLVRQYNPKMVKVASLLVKRTPRSVGYKPDFVGF
                ..**..*..  .              .     . *           .

M.avium         DIPNDFVVGYGLDYDERYRDLSYIGTLDPRVYQQ----------------
M.tuberculosis  DIPKTFVVGYGLDYDERYRDLSYIGTLDPRVYQ-----------------
V.harveyi       AIPDEFVVGVGIDYAQKYRDLPFIGKVVP---QE----------------
L.donovani      TIPHAFVIGYGMDYAESYRELRDICVLKKEYYEKPE--------------
T.brucei        DVPNVFVVGYGLDYDQSYREVRDVVILKPSVYETWGKELERR-KAAG---
T.cruzi         NIPNAFVIGYGLDYDDTYRELRDIVVLRPEVYAEREAARQKKQRAIGSAD
P.falciparum    SIPDHFVVGYSLDYNEIFRDLDHCCLVND---------------------
C.fasciculata   TIPHAFVIGYGMDFAEAYRELRDVCVLKKEYYEKPA--------------
HUMAN           EIPDKFVVGYALDYNEYFRDLNHVCVISE---------------------
                 .*. **.* ..*. . .*..

M.avium         --------------
M.tuberculosis  --------------
V.harveyi       --------------
L.donovani      -----------SKV
T.brucei        ---E-------AKR
T.cruzi         TDRDAKREFH-SKY
P.falciparum    ---EGKKKYKATSL
C.fasciculata   -----------SKL
HUMAN           ---TGKAKYKA---
```

Figure 29. Comparison of HGPRT amino acid sequences (% identity).

Abbreviations are: *M.a.* - *Mycobacterium avium; M.t.* - *Mycobacterium tuberculosis; H.s.* - *Homosapiens sapiens; L.d.* - *Leishmania donovani; C.f.* - *Crithidia fasciculata; P.f.* - *Plasmodium falciparum; T.b.* - *Trypanosoma brucei; T.c.* - *Trypanosoma cruzi; T.g.* - *Toxoplasma gondii; T.f.* - *Tritrichomonas feteus*

HGPRTase % Identity

	M.a.	*M.t.*	*H.s.*	*L.d.*	*C.f.*	*P.f.*	*T.b.*	*T.c.*	*T.g.*	*T.f.*
M.a.										
M.t.	**88**									
H.s.	**26**	**26**								
L.d.	**39**	**39**	**29**							
C.f.	**38**	**38**	**30**	**78**						
P.f.	**29**	**29**	**48**	**23**	**24**					
T.b.	**36**	**36**	**28**	**41**	**43**	**31**				
T.c.	**45**	**44**	**35**	**56**	**56**	**32**	**52**			
T.g.	**26**	**25**	**41**	**25**	**28**	**52**	**30**	**29**		
T.f.	**42**	**41**	**32**	**33**	**34**	**29**	**33**	**37**	**26**	

Apparent K_m values of 9.8 μM and 21.7 μM for hypoxanthine and guanine respectively were determined by Hanes analysis (Figure 30 and 31). The k_{cat} values were calculated to be 1.43 s^{-1} and 1.1 s^{-1}. Isoelectric focusing of both *M. avium* and *M. tuberculosis* HGPRT resolved at least four bands for each and revealed a pI range of ≅ 4.7 - 5.1 and 4.6 - 4.9 respectively (See Figure 32).

The effect of Mg^{+2} concentration on *M. tuberculosis* HGPRT is depicted in Figure 33. Low concentrations of 0.5 to 2.0 mM allowed miniscule initial rates. A range between 5 - 40 mM $MgCl_2$ revealed a somewhat skewed bell-shaped curve with peak activity seen at 20 mM. At greater concentrations, the magnesium divalent cation became inhibitory and resulted in a linear decline in enzyme activity. Substitution of cobalt or manganese augmented HGPRT activity above that seen with magnesium (5 mM) while other cations including calcium, copper, and iron were not reasonable alternatives. Zinc allowed phosphoribosylation *albeit* in a much reduced capacity (Figure 34).

The effect of pH on the phosphoribosylation of hypoxanthine by HGPRT is shown in Figure 35. Initial reaction rates were shown to be very slow below pH 6.0 and increasingly more rapid up to pH 9.0 with the enzyme active over a wide pH range.

Figure 30. Hanes analysis of recombinant *M. tuberculosis* HGPRT.

M. tuberculosis HGPRT was assayed spectrophotometrically at 37° C with hypoxanthine. A K_m of 9.7 μM and a k_{cat} of 1.43 s^{-1} was calculated.

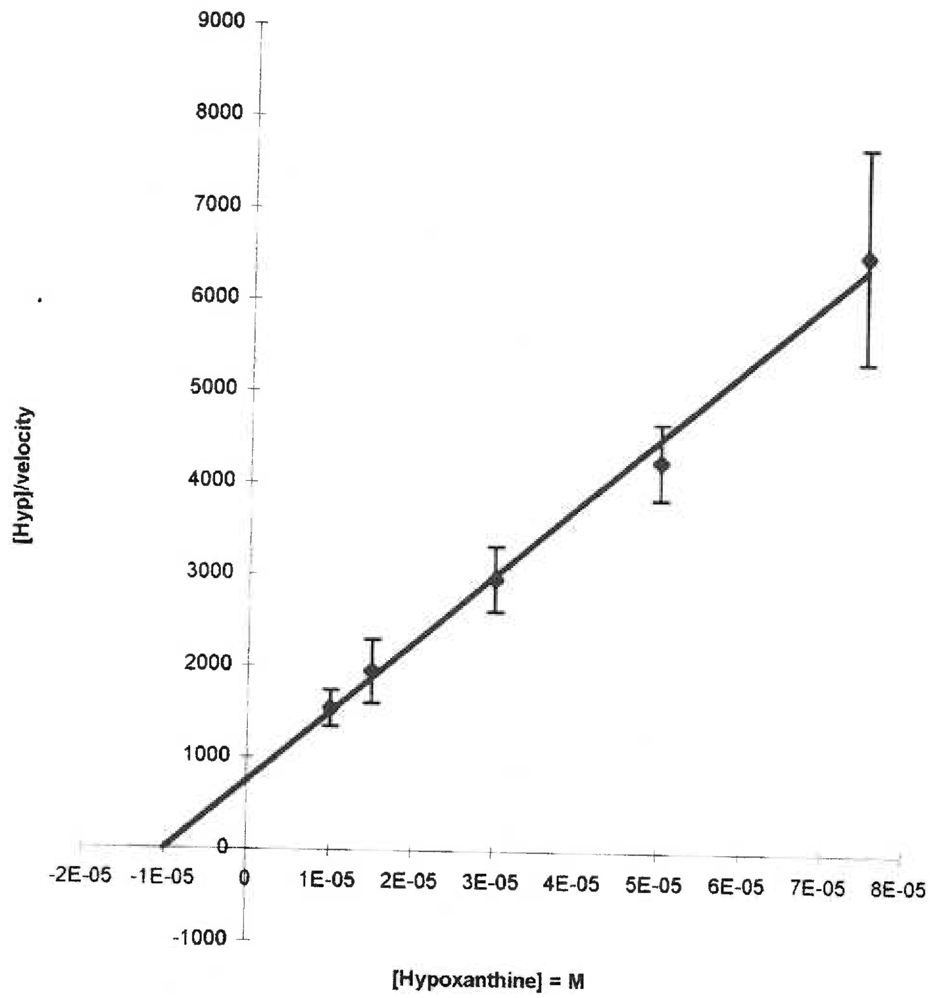
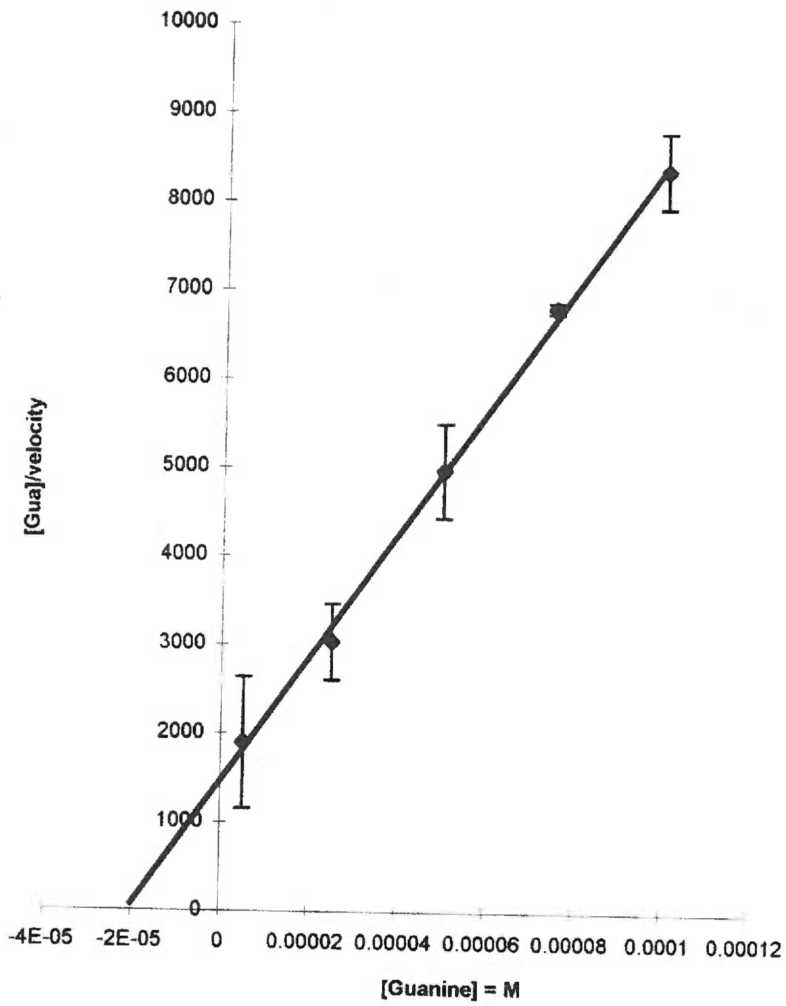

M.t. HGPRT - hypoxanthine kinetics
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
[Hyp]/velocity
-2E-05
-1E-05
0
1E-05
2E-05
3E-05
4E-05
5E-05
6E-05
7E-05
8E-05
[Hypoxanthine] = M

Figure 31. Hanes analysis of recombinant *M. tuberculosis* HGPRT.

M. tuberculosis HGPRT was assayed spectrophotometrically at 37° C with guanine. A K_m of 27.1 µM and a k_{cat} of 1.1 s^{-1} was calculated.



Mt. HGPRT - guanine kinetics
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
[Gua]/velocity
-4E-05
-2E-05
0
0.00002
0.00004
0.00006
0.00008
0.0001
0.00012
[Guanine] = M

Figure 32. Isoelectric focusing of purified *M. avium* and *M. tuberculosis* HGPRT.

ISOELECTRIC FOCUSING GEL

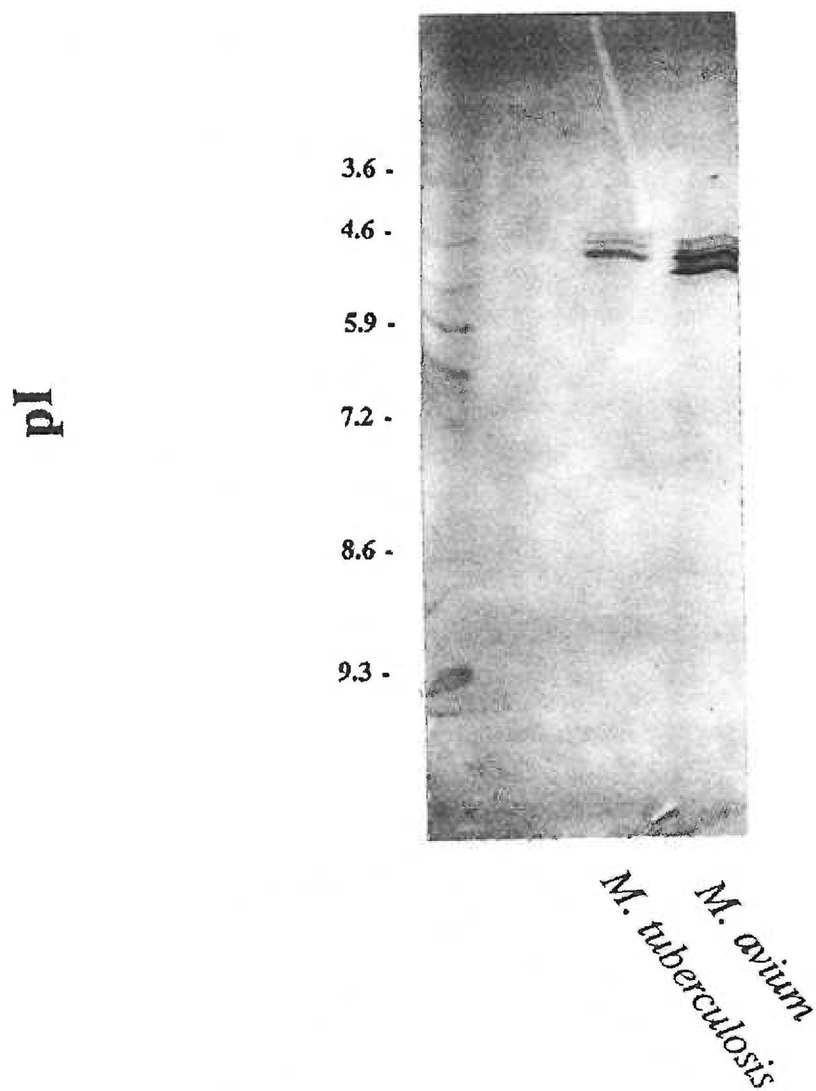
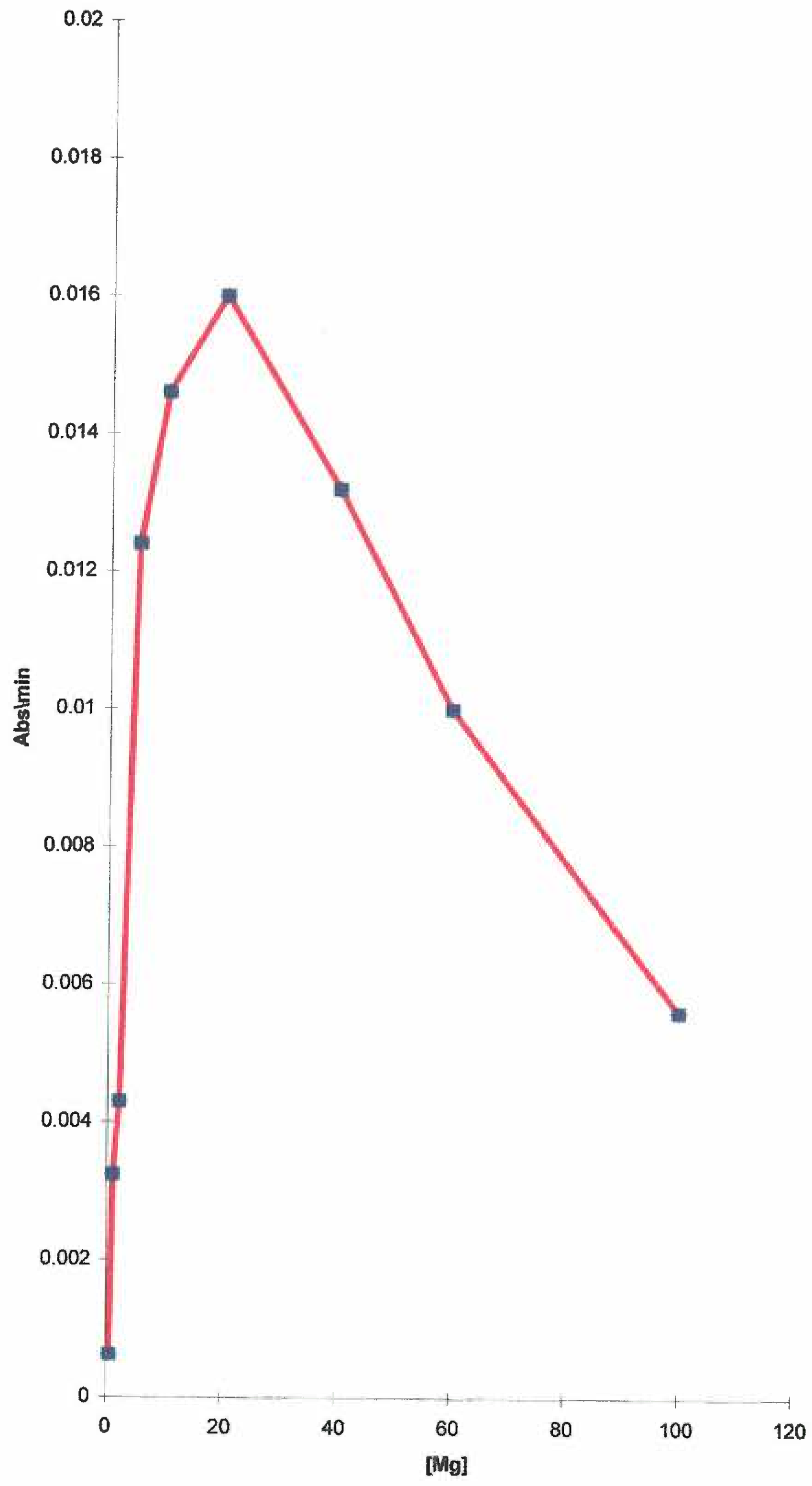


Figure 33. Magnesium effect on HGPRT.

The effect of varying magnesium concentration on the initial velocity of the hypoxanthine phosphoribosylation reaction. All conditions were as described in "Material and Methods" other than the change in $MgCl_2$ concentration.



Effect of Mg on HGPRT initial rate
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Abs\min
0
20
40
60
80
100
120
[Mg]

Figure 34. Cation effect on HGPRT.

Effect of substitution of various 5 mM cations for magnesium. Radiolabeled assays utilizing hypoxanthine were performed as described in "Materials and Methods".

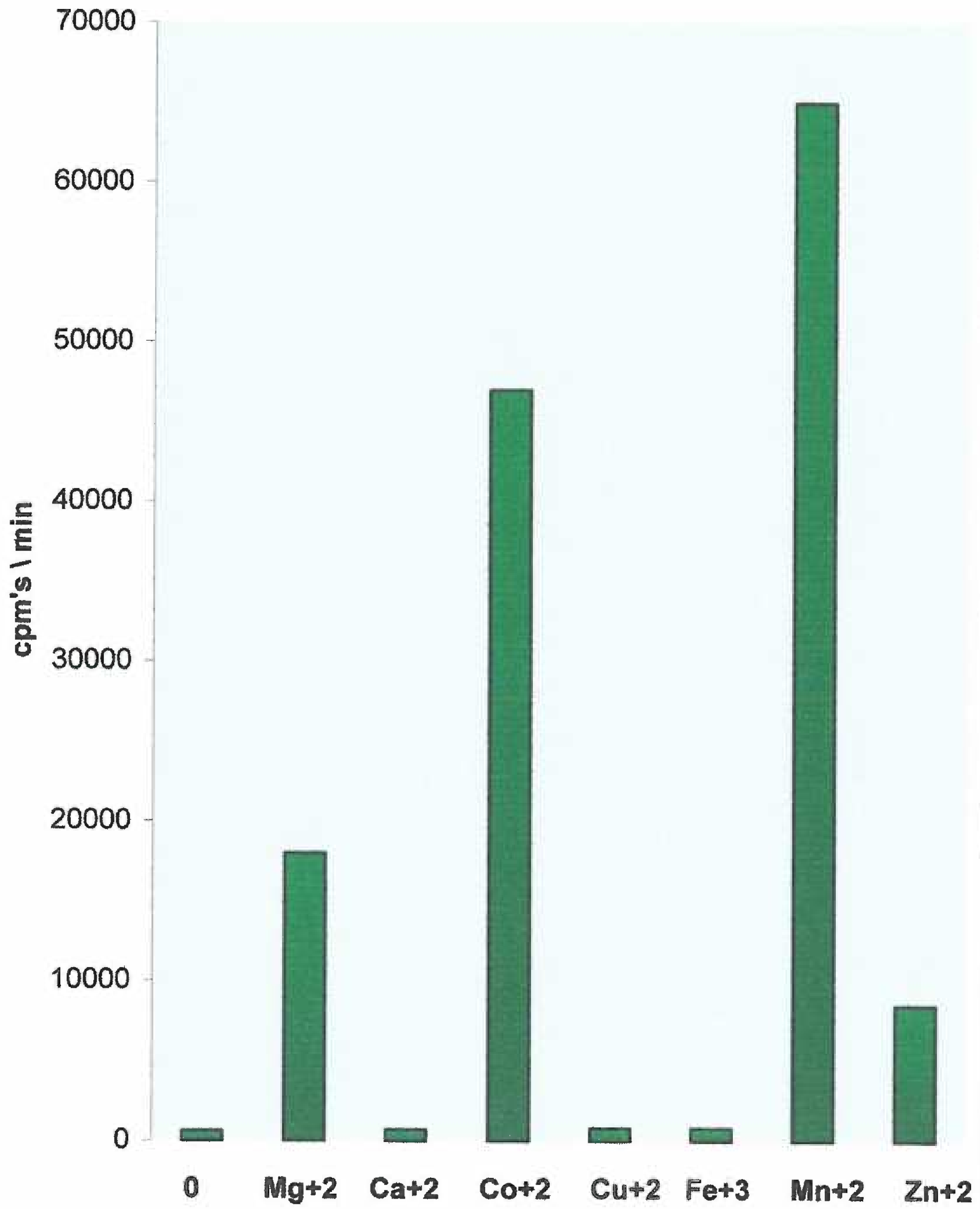
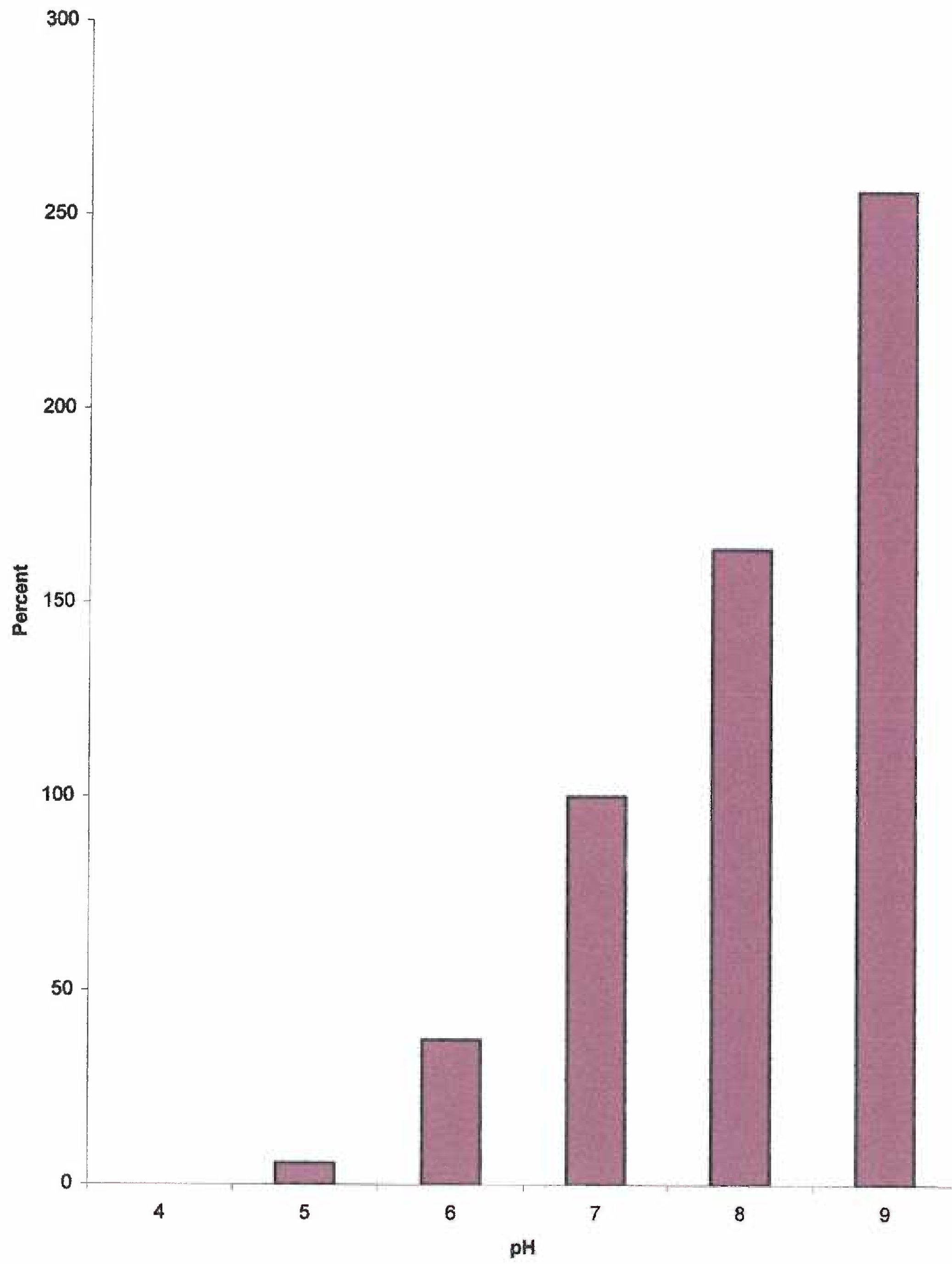

Cation Effect on HGPRT
70000
60000
50000
40000
30000
20000
10000
0
cpm's \ min
0
Mg+2
Ca+2
Co+2
Cu+2
Fe+3
Mn+2
Zn+2

Figure 35. Effect of pH on HGPRT.



pH Effect
300
250
200
150
100
50
0
Percent
4
5
6
7
8
9
pH

Crystallization of the *M. tuberculosis* HGPRT: Completion of Crystal Screens I and II (Jancarik and Kim, 1991),(Hampton Research, Laguna, CA) using the vapor diffusion-hanging drop method resulted in the production of predominantly amorphous precipitates and/or generation of salt crystals. Half-screens allowed the creation of small hexagonal bipyramidal crystals by mixing crystallization reagent 1:1.2 (v:v) with 10mg/ml HGPRT over a 0.75 ml reservoir of 2.0 M sodium formate containing no buffer or salt. Empirical optimization resulted in hexagonal bipyramidal crystals grown to 0.28 x 0.14 mm over two months (room temp) in 2.5 M sodium formate by vapor diffusion sitting drop conditions (Figure 36). The ratio of reagent to protein remained consistant but the total volume of the drop increased to 40 µl. Analysis of still diffraction photos produced using an Enraf-nonius FR590 sealed tube x-ray generator determined that the crystals diffracted to greater than 4 Å and were not data collection quality.

Purine analog screens against *M. avium* and *M. tuberculosis*: Twenty-two cryptic purine analogs from Burroughs Wellcome (Beckenham, UK) and seven others purchased from Sigma Chemical Co. (St. Loius, MO) were tested against both *M. avium*, strain #101, and *M. tuberculosis,* Erdman, in culture (See Table 1, Appendix). After one month in the presence of 0.12 mg/ml drug, only one and eight of 29 were capable of inhibiting culture growth for *M. avium* and *M. tuberculosis* respectively. The initial screen showed that 6-mercaptopurine

Figure 36. Crystallization of *M. tuberculosis* HGPRT.

Hexagonal bipyramidal crystals of *M. tuberculosis* HGPRT grew to 0.28 x 0.14 mm in 2.5 M formate precipitant, without buffer or salts over a period of 2 months at room temperature using vapor diffusion sitting drop conditions. This crystal diffracted to ≅ 4.0 Å.

Mycobacterium tuberculosis HGPRT

was effective against both *M. avium* and *M. tuberculosis* while 6-cyanopurine; 2-amino, 6-isothioaminopurine; 2-amino, 6-thiocyanopurine; 2-amino, 6-iodopurine; azathiopurine (Imuran); and 6-nitrosomethylpurine suppressed *in vitro* growth of *M. tuberculosis* as determined by visual inspection of each tube compared to a negative control (no mycobacteria added) and a positive control (no drug added) (See Figure 37).

The secondary purine analog screen (data depicted in Figure 38) consisted of essentially the same protocol except that only the effective drugs from the primary screen were diluted serially starting at a concentration of .06 mg/ml. Six concentrations of each analog were tested (growth was checked at one month) and were labeled A-F with A the most concentrated (.06, .03, .015, .0075, .00375, .001875 mg/ml final concentration). *M. avium* showed no growth inhibition in the presence of 6-mercaptopurine at any concentration. Therefore, the effective concentration of 6-mercaptopurine inhibition is ≤ 0.12 and > 0.06 mg/ml. Three drugs effectively prevented growth of *M. tuberculosis* at the lowest concentration tested (F), i.e. 0.00375 mg/ml. They included: 2-amino,6-thioaminopurine; 6-iodopurine and 6-nitrosomethylpurine. The other five drugs were inhibitory for growth in decreasing order as follows: 2-amino,6-iodopurine (E); 6-mercaptopurine (D); 2-amino,6-thiocyanopurine (D); 6-cyanopurine (C); azathiopurine (C). After two months in culture, the drugs remained effective

Figure 37. Primary *in vitro* drug screens of purine analogs against *M. avium* and *M. tubercuosis.*

Seven purine analogs from Sigma (St. Loius, MO) and twenty-two from Dr. Rich Miller, Burroughs Wellcome (Beckenham, UK) were designated 1-7 and 8-29 (original designations shown in green) and dissolved in 0.05N NaOH to a final concentration of 3 mg/ml. One ml cultures containing 0.12 mg/ml (≅ 800 μM for each) and either 1.15×10^6 CFU of *M. avium* or 2.5×10^5 CFU *M. tuberulosis* were incubated for one month in the presence of drug or no drug (positive control). A negative control without mycobacteria added was included in the experiment. Visual inspection for growth was recorded. A (+) indicates growth as compared to the positive control and a (-) indicated the lack of mycobacterial proliferation consistant with the negative control containing no organisms.

Primary Drug Screen against *M. avium* and *M. tuberculosis*.

Tube #	Drug		*M.a*	*M.t.*
1.	8-azahypoxanthine		+	+
2.	8-azaxanthine		+	+
3.	allopurinol		+	+
4.	2-thioxanthine		+	+
5.	4-mercapto, 1-*N*-pyrazolopyrimidine		+	+
6.	8-azaguanine		+	+
7.	6-thiopurine		-	-
8.	2-amino, 6-thiomethylpurine	(1085U54)	+	+
9.	6-cyanopurine	(0298U54)	+	+
10.	7-deaza-8-azaguanine	(0158U56)	+	+
11.	Imuran (hypoxanthine)	(0322U57)	+	+
12.	8-methylpurine	(0239U59)	+	+
13.	2-aminoethylpurine	(0256U55)	+	+
14.	2-amino, 8-phenylpurine	(0303U49)	+	+
15.	6-thiocyanopurine	(0041U55)	+	-
16.	2-amino, 6-thiopurine	(0207U49)	+	+
17.	6-carboxyaminopurine	(0017U55)	+	+
18.	2-amino, 6-isothioaminopurine	(0022U59)	+	-
19.	6-sulfoxymethylpurine	(0318U60)	+	+
20.	9-deaza-8-axahypoxanthine	(0055U64)	+	+
21.	2-amino, 6-thiocyanopurine	(0213U59)	+	-
22.	2-amino, 6-iodopurine	(0022U59)	+	-
23.	6-oxyphenylpurine	(0346U52)	+	+
24.	2-amino, 8-methylpurine	(0130U49)	+	+
25.	6-carboxypurine	(0001U55)	+	+
26.	8-thiopurine	(0055U52)	+	+
27.	Imuran (guanine)	(0323U57)	+	-
28.	6-iodopurine	(0300U53)	+	-
29.	6-nitrosomethylpurine	(0253U57)	+	-
30.	Positive control (no drug)		+	+
31.	Negative control (no mycobacteria)		-	-

Figure 38. Secondary *in vitro* drug screens of purine analogs against *M. avium* and *M. tubercuosis.*

A. One and eight purine analogs found to be inhibitory to *M. avium* and *M. tuberculsois* growth respectively at 0.12 mg/ml were retested at lower concentrations serially diluted in liquid culture. The concentrations are labelled A-F representing decreasing amounts and are 0.06, 0.03, 0.015, 0.0075, 0.00375 and 0.001875 mg/ml. (The approximate molar amounts range from 400 - 10 μM.) Visual inspection occurred one month (light blue) and six weeks (royal blue) following initial set up. A (+) indicates growth and (-) symbolizes a lack of growth.

B. Aliquots (100 μl) from cultures determined to have an inhibitory effect were plated onto 10 cm Middlebrook 7H11 agar plates and incubated for one month to test whether the lack of growth in culture was bacterialcidal or inhibitory only (did not kill mycobacteria, only prevented proliferation under the drug conditions). Symbols (red) described above indicate results.

Secondary Drug Screen against *M. avium* and *M. tuberculosis.*

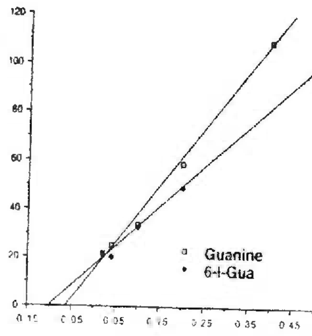
Tube #	Drug	A	B	C	D	E	F
Mycobacterium avium							
7.	6-thiopurine	+	+	+	+	+	+
Mycobacterium tuberculosis							
7.	6-thiopurine	-	-	-	-	+	+
		-	-	-	+	+	+
	plate assay			+	+		
15.	6-thiocynopurine	-	-	-	+	+	+
	(0041U55)	-	-	+	+	+	+
	plate assay		+	+			
18.	2-amino, 6-isothioaminopurine	-	-	-	-	-	-
	(0022U59)	-	-	-	-	-	-
	plate assay					-	-
21.	2-amino, 6-thiocyanopurine	-	-	-	-	+	+
	(0213U59)	-	-	-	+	+	+
	plate assay			+	+		
22.	2-amino, 6-iodopurine	-	-	-	-	-	+
	(0022U59)	-	-	-	-	-	+
	plate assay			-	+	+	
27.	Imuran (guanine)	-	-	-	+	+	+
	(0323U57)	-	-	+	+	+	+
	plate assay		+	+			
28.	6-iodopurine	-	-	-	-	-	-
	(0300U53)	-	-	-	-	-	-
	plate assay					-	+
29.	6-nitrosomethylpurine	-	-	-	-	-	-
	(0253U57)	-	-	-	-	-	-
	plate assay					-	-

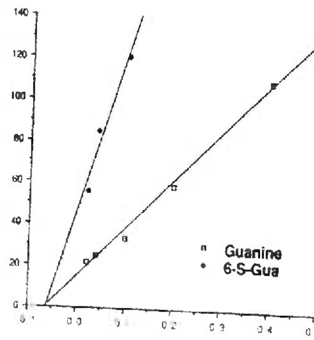
against *M. tuberculosis*. Results are as follows (also shown in Figure 34): Four drugs (2-amino,6-isothiaminopurine; 2-amino,6-iodopurine; 6-iodopurine; and 6-nitrosomethylpurine) were determined to be bacterialcidal at the following concentrations (designated as) F, C, E, F respectively. Observation of no growth after one month on 7H11 Middlebrook agar following plating of 100 μl of each culture exposed to drug, visibly negative for growth, was considered bacterialcidal. Those plates on which *M. tuberculosis* grew, indicated that growth was only inhibited in culture in the presence of drug but when drug pressure was removed, the mycobacteria thrived. Drugs inhibitory to *M. tuberculosis* were 6-mercaptopurine (at concentration C); 6-cyanopurine (B); 2-amino, 6-thiocyanopurine (B); and imuran (C).

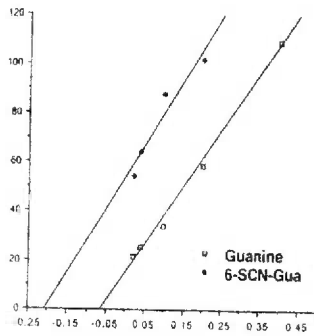
Analysis of Lineweaver-Burk plots of initial velocities for *M. tuberculosis* HGPRT in the presence of guanine or guanine and drug revealed that 2-amino, 6-thiopurine and 6-cyanopurine were competitive inhibitors (ie., a reduction in maximal velocity was seen). Mixed inhibition occurred in the presence of 2-amino, 6-cyanopurine as both V_{max} and K_m was changed. Interestingly, an atypical noncompetitive inhibition was shown for both 2-amino, 6-iodopurine and 2-amino, 6-isothioaminopurine. For each, the K_m was lowered and the V_{max} was raised (Figure 39.)

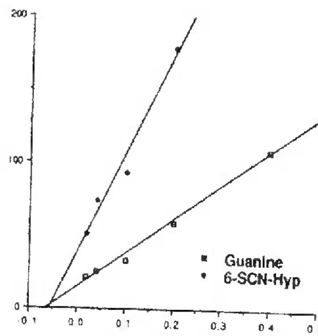
Figure 39. Lineweaver-Burk analysis of *M. tuberculosis* HGPRT.

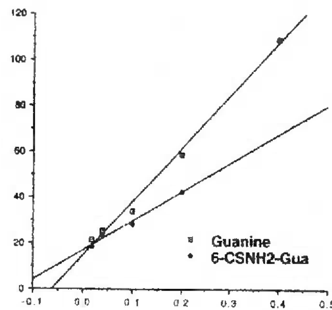
M. tuberculosis HGPRT was assayed spectrophotometrically at 37°C, pH 7.4, with guanine alone and guanine in the presence of 0.06 mg/ml purine analog. Analogs tested were (from left to right and top to bottom) 2-amino, 6-iodopurine; 2-amino, 6-thiopurine; 2-amino, 6-thiocyanopurine; 6-thiocyanopurine, 2-amino, 6-isothio-aminopurine.


Guanine
6-I-Gua


Guanine
6-S-Gua


Guanine
6-SCN-Gua


Guanine
6-SCN-Hyp


Guanine
6-CSNH2-Gua

DISCUSSION

Leishmania donovani: The cloning of the *L. donovani xprt* was undertaken to investigate the purine salvage pathway as a viable target for structure-based drug design. Previously, the *L. donovani hgprt* and *aprt* genes have been cloned and characterized (Allen et al.; 1995a, 1995b). Genetic analysis of *L. donovani* mutants created utilizing transfection (Kapler et al., 1990) and subsequent homologous gene replacement (Cruz and Beverley, 1990) of Δ*hgprt* and Δ*aprt* in adenosine kinase minus cells determined that growth in defined media containing either individual purine bases (hypoxanthine, xanthine, guanine, or adenine) or nucleosides (adenosine, inosine or guanosine) was permitted (Hwang and Ullman, 1997). These data suggested that the nucleosides were hydrolyzed to their respective bases by nucleoside hydrolase (Koszalka and Krenitsky, 1979) and adenosine hydrolase/phosphorylase (Iovannisci and Ullman, 1984) present in *L. donovani* promastigotes. Oxidative deamination of guanine to xanthine (LaFon et al., 1982) is a logical explanation for the observed growth in media containing guanosine and guanine as phosphoribosylation of xanthine by XPRT would allow production of guanylate nucleotides and eventual conversion to adenylate nucleotides (see Fig. 2), thereby allowing growth. However, the ability of the null mutants to grow on hypoxanthine, adenine, inosine and adenosine led to the postulation that another enzyme, possibly XPRT,

had the capacity to convert them in some form to usable energy and overcome the genetic lesions of HGPRT$^-$, APRT$^-$, and AK$^-$. Consequently, *xprt* was cloned to initiate studies to determine its importance in the *L. donovani* purine salvage pathway. As humans do not have this enzyme, it represents an ideal target for chemotherapeutic exploitation in drug development for visceral leishmaniasis.

The *xprt* cDNA was isolated from an *L. donovani* directional λZAPII library (gift of Dr. Ken Stuart, Seattle Biomedical Research Institute, Seattle, WA). The capacity of the λZAPII library to be *in vivo* excised forming pBluescript plasmids with *L. donovani* cDNA inserts, permitted functional complementation in purine auxotrophic Sϕ609 *E. coli* cells. The Sϕ609 *E. coli* cells containing pBS-*xprt* allowed growth of individual colonies on LPI-agarose plates supplemented with xanthine while other cDNA containing pBS vectors did not. Sequence analysis supported the identification of the clones as containing a phosphoribosyltransferase.

The full length genomic *xprt* has been isolated by utilizing the cDNA clone as a hybridization probe to screen a genomic cosmid *L. donovani* library. Nucleotide sequence analysis revealed an open reading frame of 241 amino acids and the ubiquitous 5' spliced leader mini-exon (Borst, 1986) was used to help determine a start site. Several lines of evidence suggest that the initiating codon for methionine and the open reading frame is accurate for *xprt*. The putative start

site described is the first AUG following the defined splice leader site. No in-frame stop sites are seen until after the three codons encoding the AKL-COOH glycosomal targeting signal. The other two frames contain several stops. And most significantly, the open reading frame ascribed to the *xprt* gene is consistent with published G-C bias for *Leishmania* (Alvarez et al., 1994). An 82.3 % G-C content is seen within the 3rd codon position throughout the open reading frame while immediately adjacent to the region a decrease to 58.5% is seen. An overall total of 60 % G or C was found. Ultimately, a subclone of the gene expressed in Sϕ609 *E. coli* complemented the genetic *(x)prt* lesion on xanthine containing LPI media and overproduced a protein of 30,000 Daltons, apparent molecular mass, which when purified to apparent homogeneity showed XPRT activity.

A 50% homology between the primary structures of *L. donovani* XPRT and HGPRT (Allen et al., 1995a) was calculated. A determination of whether the putative *xprt* was also capable of functional complementation of the Sϕ609 *E. coli* lesion on other purine sources revealed that only extended incubation permitted growth on both guanine and hypoxanthine. Consequently, the gene encoded a predominantly xanthine utilizing phosphoribosyltransferase and was thus named *xprt*. Southern analysis also determined that the *L. donovani xprt* and *hgprt* genes were in close proximity; i.e., encoded on the same 4.5 kb *EcoRI* fragment. Together, the significant homology and the nearby location suggest a primordial

gene duplication event.

Analysis of the *L. donovani xprt* predicted a 241 amino acid protein with a pI of 7.4. The calculated isoelectric point is consistent with that determined in *L. donovani* promastigotes by Tuttle and Krenitsky (1980). The enzyme was shown to contain 14 highly conserved residues and a homologous 13-residue PRPP binding motif in common with other HGPRTs including; human (Wilson et al., 1983), *Toxoplasma gondii* (Donald et al., 1996), *Crithidia faciculata* (Jiang et al., 1996), *L. donovani* (Allen et al., 1995a), *Trypanosoma brucei* (Allen and Ullman, 1993), *Trypanosoma cruzi* (Allen and Ullman, 1994), *Tritrichomonas foetus* (Chin and Wang, 1994), *Vibrio harveyi hprt* (Showalter and Silverman, 1990), *E. coli* XPRT (Pratt and Subramani, 1983), *Giardia lamblia* GPRT (Sommer and Wang, 1996) and *Lactococcus lactis hprt* (Nilsson and Lauridsen, 1992). The primary structure of PRTases has been suggested to be relatively unimportant from a catalytic standpoint and only 7 completely conserved residues predominate a comparison between a combination of hypoxanthine, guanine, and xanthine phosphoribosyltransferases (Sommer and Wang, 1996). Four short regions of homology are interspersed between long stretches of relatively unrelated sequence (Schumacher et al., 1996) (Carter and Ullman, 1995). The regions have been identified to consist of: 1) a ribose phosphate binding motif; 2) a flexible loop that protects the oxocarbonium intermediate from nucleophilic

attack by solvent; 3) a PRPP binding region; and 4) an active site domain that associates with the purine ring. The similarity is much stronger at the tertiary structural level between 'type I' PRTases including HGPRT, XPRT, orotate phosphoribosyltransferase (OPRT) and glutamine-PRPP amidotransferase (GAT) and consists of a core fold of five-stranded β sheet surrounded by three to four α helices (Eads et al., 1997; Vos et al., 1997). Clearly, not all PRTases require either the conserved tertiary structure or the PRPP binding motif. Recently, the quinolinic acid phosphotransferase (QAPRT), devoid of the conserved PRPP region, has been solved to 2.8 and 3.0 Å resolution with bound QA and nicotinic acid mononucleotide (NAMN) and was found to have a novel fold formed by a seven-stranded α/β-barrel domain (Eads et al., 1997).

The predicted primary sequence of *L. donovani* XPRT seems to reveal no clues regarding substrate specificity. The highly conserved A67 corresponds to A50 in the human enzyme where it resides in the first α helix. To date, no studies have been undertaken to assertain its role in enzyme function. Most known 'type I' phosphoribosyltransferases contain this residue, including OPRT and GAT while *Vibrio harveyi hprt* (Showalter and Silverman, 1990), *Lactococcus lactis hprt* (Nilsson and Lauridsen, 1992) *M. avium hgprt* and *M. tuberculosis hgprt* have a glycine at the same relative position and *Schistosoma mansoni* a serine (Craig, 1988).

Portions of the 'type I' $\beta 2$ - $\alpha 2$ region that surround the ribose phosphate moiety of product in solved HG(X)PRT's (but not OPRT and GAT) are conserved at the primary level. The *L. donovani* XPRT L92, G94 and F98 are consistent. Interestingly, the *E. coli* XPRT and *G. lamblia* GPRT have substitutions of (S for L, P for F), and (Y for F) respectively.

A conserved S-Y dyad is also present in *L. donovani* XPRT at positions 122-123. The dyad is contained within a region which has been determined to form an important flexible loop in the *Toxoplasma gondii* HG(X)PRT crystal structure (Schumacher et al., 1996) purportedly as protection for the extremely reactive oxocarbonium intermediate (Eads et al., 1994; Tao et al., 1996). Mutations of the Ser^{95}-Tyr^{96} dipeptide in *L. donovani* HGPRT dramatically reduced enzyme activity and confirmed an importance of Tyr^{96} for catalysis (Jardim and Ullman, 1997). Alignments of known APRTs reveal that most, but not all, also contain this dyad (Allen et al., 1995b). Since APRT crystal structures have not been solved and published, it remains to be seen if they will be a 'type I' PRTase or have a novel tertiary structure.

The next region of homology seen in the XPRT is within the PRPP binding motif. Ten different PRTases use PRPP to catalyze the formation of purine, pyrimidine, and pyridine nucleotides and in bacteria and lower eukaryotes, the formation of histidine and tryptophan (Musick, 1981). The PRPP motif is the

only region conserved among this highly divergent group of enzymes. Two adjacent acidic residues are surrounded by hydrophobic ones, followed by smaller amino acids, usually including a glycine, in all members of this large group of PRTases. The *L. donovani* XPRT PRPP binding motif exhibits conservation as well as divergence; its primary sequence is: 147-KHVLIVEDVCDSGRTL-162. Most noteworthy is the cysteine at position 156. All other HG(X)PRTs have either a valine or isoleucine at this corresponding position. Also of interest is valine 155. Although it is a conservative substitution for isoleucine, only four other species deviate; *G. lamblia* GPRT, *E.coli* XPRT, *M. avuim* and *M. tuberculosis* HGPRTs. XPRT positions L150, E153 and D154 are highly conserved with complete conservation of D175 and T161. Only three other completely conserved amino acids appear in XPRT. They include R221, G211 as well as K187 which corresponds to the human K165 postulated to be important for substrate specificity. Eads et al. (1994) showed that purine exocyclic O6 forms a hydrogen bond with the K165 and they postulated that this amino acid appeared to be critical for the selectivity of guanine and hypoxanthine over adenine.

The purine ring binding portion of the active site is relatively conserved across species. The primary sequence usually consists of [F,(V/I/L), (V/I), G*, Y, G, L, D*, (Y/F)]. The asterisks denote conservation where a deviation would be

considered unusual. The *L. donovani* XPRT sequence in this area is YIVGYGFEY. The F, which participates in aromatic-aromatic interactions with the purine ring, to Y208 is not unusual as a change at this relative position is seen in all xanthine catalyzing PRTs except *P. falciparum* HG(X)PRT. However, *L. donovani* XPRT is the only known PRT to deviate from D193 of the human sequence regardless of substrate specificity. Mutagenic analysis is currently being initiated to determine the role of the *L. donovani* XPRT E215 at this position.

The C-terminus of the *L. donovani* XPRT ends in a tripeptide sequence characteristic of glycosomal targeting (Blattner et al., 1992; Sommer et al., 1992). The Ala-Lys-Leu present is consistent with the degenerate topogenic signal necessary to import proteins into the glycosome. The *L. donovani* HGPRT also contains a glycosomal signal, Ser-Lys-Val, and has been shown by immunohistochemistry and immunoelectronmicroscopy to be selectively localized to the glycosome (Ullman et al., 1996). The cellular location of XPRT will be determined using polyclonal antibodies (Abs). Although Abs have already been produced by injection of rabbits with purified recombinant XPRT isolated from a PAGE gel, they remain to be fully characterized.

The ability of the *L. donovani xprt* to complement the Sϕ609 *E. coli* genetic lesions allowed determination of substrate specificities and production of large quantities of recombinant XPRT in the absence of the bacterial counterparts.

Xanthine was found to be readily phosphoribosylated by XPRT while adenine was not. Limited catalysis of hypoxanthine and guanine was also shown and could explain the ability of the *L. donovani* Δ*hgprt,* Δ*aprt* and ΔAK cells to grow in defined media containing either hypoxanthine, adenine, inosine and adenosine as a sole purine source. As XPRT can be used as a positive or negative selectable marker, site-directed mutagenesis of selected structural determinants can easily be tested; as can cytotoxic substances that might act as potential drugs against visceral leishmaniasis.

The cloning of the gene and overproduction of XPRT from *L. donovani* is just the beginning of the thorough molecular and biochemical investigation necessary for employing structure-based drug design. A detailed kinetic analysis of the HGPRT from human has recently been completed (Xu et al., 1997) and needs to be accomplished with the *L. donovani* XPRT. Ultimately, the XPRT structure should be solved at the anatomic level and these studies have been initiated by Dr. Armando Jardim (Ullman lab) in collaboration with Dr. Richard Brennan (OHSU; Portland, OR). To further study the purine salvage pathway of *L. donovani,* knock out mutations of XPRT need to be completed. In combination with the current knock outs of Δ*hgprt* and Δ*aprt* in ΔAK *L. donovani* promastigotes (Hwang and Ullman, 1997), the importance of these four purine salvage enzymes in survival of the parasite *in vivo* can now be elucidated.

As the salvage pathway plays a crucial nutritional role in *Leishmania donovani*, it seems reasonable to expect that the parasite should be vulnerable to key salvage enzyme inhibitors or subversive substrates. Timely discovery is of the essence as multiple drug resistance continues to develop while chemotherapeutic choices are limited and toxic.

Mycobacteria: Although purine salvage has been studied in some detail in humans, a dearth of knowledge remains about the general pathway for mycobacteria. The ability to grow in chemically defined minimal media (devoid of purines) infers the existence of an intact purine biosynthetic pathway in both *M. avium* and *M. tuberculosis,* qualifying them as purine prototrophs. Only *M. leprae*, to date, has been unable to be cultivated in defined minimal media, suggesting that this mycobacterial species, unlike others, may indeed be purine auxotrophic (Wheeler, 1987a). In a limited study involving *M. avium*, purine biosynthetic and salvage enzymes were found to be only weakly metabolically regulated (Wheeler, 1987b) suggesting an important role for each pathway regardless of energetic demand.

Direct attempts to clone mycobacterial genes of known enzymes have been met with only limited success and the cloning of the *hgprt* gene from both *M. avium* and *M. tuberculosis* now provides the opportunity to study their purine salvage pathway at the genetic, biochemical, and physiological level. The

apparent lack of the ability of mycobacteria to regulate purine metabolism poses *hgprt* as an exciting potential therapeutic target for the development of novel antimycobacterialcidal agents. As mentioned earlier, unique considerations must be met in the design of drugs expected to be active against these organisms. The limited porosity of their cell envelope must be overcome. Another barrier is their intracellular location in the macrophage endosomal compartment. Purine analogs have a potential for entry through these two selectively permeable barriers as natural purine transporters appear to allow the entry of structural analogs. Phosphoribosylation functions to "trap" the drug within the mycobacterium and eventually leads to its selective demise. Further work to characterize and understand the entire pathway is needed. An additional aspect of utmost importance is the accurate determination of salvageable purine levels within the macrophage. I have not found a single report in the literature describing such measurements. Concentrations measured in blood and within oocytes have been used to extrapolate that either hypoxanthine or adenine nucleotides are the most prevalent source respectively (Ullman and Carter, 1995; Wheeler, 1987b). Blood concentrations could be significantly different from those within the macrophage due to the presence of xanthine oxidase in endothelial cells lining blood vessels (Moriwaki et al., 1993). Xanthine oxidase oxidizes hypoxanthine and xanthine to uric acid for elimination. It can easily be argued that the specialized

metabolism in an oocyte is not particularly applicable to that of a macrophage. Consequently, the cloning of the *M. avium* and *M. tuberculosis hgprt* genes is only an initial step of many that need to be completed.

Due to the characteristic of mycobacterial DNA to have an extremely high G-C content, cloning by cross hybridization to other known *hgprt* genes was not attempted. A PCR-based strategy was exploited to amplify a specific *hgprt* fragment from *M. avium* using degenerate oligonucleotides primers created to both the highly conserved PRPP and purine ring binding regions near the carboxy- terminus. Full length *hgprt* genes from both *M. avium* and *M. tuberculosis* were isolated from genomic libraries using the *M. avium hgprt* PCR fragment as a probe. Each was represented as a single copy gene.

A modified method of dideoxy-chain termination DNA sequencing was developed to allow nucleotide analysis of the mycobacterial DNA with its high G-C content. Several open reading frames were found and an empirical approach was taken to elucidate the likely initiating codon for each *hgprt* gene. PCR amplification with specific oligonucleotides allowed the subclone of each possible gene sequence into the pBAce vector, followed by transformation into Sϕ609 *E. coli* and complementation selection on hypoxanthine and/or guanine. The ability to complement the genetic lesions of the Sϕ609 *E. coli* was taken as evidence of our assignment of a correct start site and in-frame coding region. Among the

several possible, two homologous GTG codons in each gene conferred phosphoribosylation activity (Figure 25) and are considered likely to be correct. No further attempt was made to determine which of the two feasible sites was utilized *in vivo* and the adjacent region did not seem to reveal a Shine Delgarno sequence. An extremely high propensity for a G or C in the 3rd position of codons was found, further evidence that either of the two start sites were consistent with a correct reading frame. The potential for the presence of a protein intron (Perler et al., 1992; Shub and Goodrich-Blair, 1992; Davis et al., 1994) was considered and dismissed as no conserved hexapeptide splice site was coded for in any reading frame.

Nucleotide sequence analysis revealed that the mycobacterial *hgprt* genes were highly related with an 88% identity between them while exhibiting limited identity to *hgprt* genes from other species (see Figure 28). The *M. avium* and *M. tuberculosis hgprt* polypeptides were deduced to be 203 and 202 amino acid respectively. The calculated molecular mass for each was 24.0 and 23.9 kD while the recombinant proteins were found to migrate with an apparent molecular size of ≅27.0 kD on 15% PAGE-gels. The discrepancy is believed to be due to altered mobility due to acidic residues rather than a change in size resulting from posttranslational modifications. Isoelectric focusing determined pI values of approximately 4.8 for each.

The mycobacterial HGPRT's primary structures are similar to others which have been described. Only four regions of homology are seen in a comparison between others with long stretches of dissimilarity intervening. Again, these regions are: 1) a ribose phosphate binding motif; 2) a flexible loop that protects the oxocarbonium intermediate from nucleophilic attack by solvent; 3) a PRPP binding region; and 4) an active site domain that associates with the purine ring. Based on this relatedness, a tertiary structural "type I" PRTase fold is predicted.

Enzymological determinations employing homogeneously purified recombinant HGPRT from the Sϕ609 *E. coli* cells genetically deficient in the corresponding PRT genes substantiated complementation results that hypoxanthine and guanine are natural substrates of the enzymes while xanthine and adenine are not. Thus, *M. avium* and *M. tuberculosis* HGPRT displays a substrate specificity similar to the human enzyme (Wilson et al., 1983). And like the human enzyme, a higher affinity for hypoxanthine over guanine is seen. However, the mycobacterial enzyme shows a marked decrease in affinity for both and a much slower turnover (Xu et al., 1997). (Hypoxanthine and guanine K_m values are 0.45 μM vs. 9.8 μM and 3.5 μM vs. 21.7 μM for human vs. mycobacteria respectively. Hypoxanthine and guanine k_{cat} values are 6.0 and 13.0 s^{-1} vs. 1.43 and 1.1 s^{-1}.)

The effect of varying magnesium concentration on HGPRT initial velocity

reveals a skewed parabolic curve with a sharp rise and gentle fall and is similar between the human and *M. tuberculosis* HGPRT. Only the apex differs slightly with peak magnesium concentrations at 40 mM and 20 mM, respectively. An E-D dyad in the active site which has been suggested to stabilize the partial positive charge of the oxocarbonium intermediate, has been implicated in Mg^{+2} binding through the structure determination of the *Toxoplasma gondii* HG(X)PRT in its apo and product forms (Schumacher et al., 1996). These authors postulate that Mg^{+2} binding may form a steric barrier to substrate binding, the formation of enzyme-product complex or catalytically induced conformational changes.

Crystallization studies in collaboration with Dr. Richard Brennan (OHSU), were initiated with the *Mycobacterium tuberculosis* HGPRT. Unfortunately, hexagonal bipyramidal crystals that formed in 2.5 M formate were not of data collection quality. Further screening needs to be undertaken as the solved structure of the *M. tuberculosis* HGPRT would be a powerful tool in structure-based design computer protocols, especially given the available data on the human enzyme.

Significant progress in the initial screening of mycobacteria against purine analogs has been made. Several compounds with substitutions at the exocyclic 6-position have been shown to be effective either in inhibiting growth or killing of *Mycobacterium tuberculosis in vitro.* The same drugs were not active against

M. avium. At least two explanations are plausible. 1) The starting concentration of the cultures was almost five times greater than the *M. tuberculosis*; therefore allowing *M. avium* to overcome the drugs. Mutation events, secretion of an inactivator, or selective metabolism to a non-toxic compound are all possible. 2) The most likely interpretation is that the *M. avium* bacteria are inherently more resistant to penetration by the purine analogs.

It has not been determined conclusively if the actions of the purine analogs against *M. tuberculosis* are specific to HGPRT or if multiple sites of action are responsible. Three analogs were active at the lowest concentration tested and need further characterization to determine their minimal effective dose. Given the magnitude of need for the development of new, effective chemotherapeutica for treatment of TB and for countering the emerging drug resistance phenomenon, work in this area is both timely and important.

CONCLUSION

The existence of lead compounds that act as inhibitors or subversive substrates of the HGPRT enzymes from *Leishmania donovani, Mycobacterium avium* and *Mycobacterium tuberculosis* and the uniqueness of the XPRT protein also from *Leishmania donovani* make these enzymes attractive possible targets for antiparasitic chemotherapy. The ease of production of recombinant protein in the genetically deficient Sϕ609 *E. coli* greatly facilitates our ability to study their specificity, kinetic mechanisms and catalytic capacities. Comparison to the human counterpart, HGPRT, is made facile by correlation to the extensive investigations of recent years (Eads et al., 1994; Xu et al., 1997). Solved anatomic structures of each enzyme will allow docking experiments utilizing computerized screening for potential lead compounds. Ultimately, I see the work described in my thesis as leading to the construction of computer models of the three enzymes. This is necessary to allow for faithful "docking experiments" to speed our quest for safe and effective treatments for each of these devastating parasitic infections.

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APPENDIX

TABLE I.

Drugs used in Screen against *M. avium* and *M. tuberculosis*.

Tube #	Drug	Burroughs Wellcome #
1.	8-azahypoxanthine	
2.	8-azaxanthine	
3.	allopurinol	
4.	2-thioxanthine	
5.	4-mercapto, 1-*N*-pyrazolopyrimidine	
6.	8-azaguanine	
7.	6-thiopurine	
8.	2-amino, 6-thiomethylpurine	(1085U54)
9.	6-cyanopurine	(0298U54)
10.	7-deaza-8-azaguanine	(0158U56)
11.	Imuran (hypoxanthine)	(0322U57)
12.	8-methylpurine	(0239U59)
13.	2-aminoethylpurine	(0256U55)
14.	2-amino, 8-phenylpurine	(0303U49)
15.	6-thiocyanopurine	(0041U55)
16.	2-amino, 6-thiopurine	(0207U49)
17.	6-carboxyaminopurine	(0017U55)
18.	2-amino, 6-isothioaminopurine	(0022U59)
19.	6-sulfoxymethylpurine	(0318U60)
20.	9-deaza-8-axahypoxanthine	(0055U64)
21.	2-amino, 6-thiocyanopurine	(0213U59)
22.	2-amino, 6-iodopurine	(0022U59)
23.	6-oxyphenylpurine	(0346U52)
24.	2-amino, 8-methylpurine	(0130U49)
25.	6-carboxypurine	(0001U55)
26.	8-thiopurine	(0055U52)
27.	Imuran (guanine)	(0323U57)
28.	6-iodopurine	(0300U53)
29.	6-nitrosomethylpurine	(0253U57)

ABBREVIATIONS

ADC - arginine decarboxylase

AIDS - acquired immunodeficiency syndrome

AMP -adenosine monophosphate

APRT - adenine phosphoribosyltransferase

BCG - bacille of Calmette and Guérin

CFU - colony forming unit

CoA - coenzyme A

CR - complement receptor

DAT - direct agglutination test

DDT - (dichloro-diphenyl-trichloro-ethane)

DHFR-TS - dihydrofolate reductase-thymidylate synthase

DMFO - DL-α-difluoromethylornithine

DNA - deoxyribonucleic acid

DOT - direct observed therapy

dTMP - deoxythymidine monophosphate

DTT - dithiothreitol

EC_{50} - median effective concentration

ECG - electrocardiogram

ELISA - enzyme linked immunosorbent assay

FAD - flavin adenine dinucleotide

FDA - Food and Drug Administration

G + C - guanine and cytosine base content of DNA, usually expressed as %.

GM-CSF -granulocyte/macrofage colony stimulating factor

GMP -guanosine monophosphate

GPI - glycosylphosphatidalinositol

GPL - glycopeptidolipid

GTP - guanosine triphosphate

HGPRT - hypoxanthine phosphoribosyltransferase

HIV - human immunodeficiency virus

IFAT - indirect fluorescence antibody test

IgG - immunoglobin G (antibody)

IgM - immunoglobin M (antibody)

IMPDH -inosine monophosphate dehydrogenase

INF-γ - interferon

INH - isoniazid

LPG - lipophoaphoglycan

LPI - low phosphate induction media

MAC - *Mycobacterium avium* complex

MDR - multiple drug resistance

NAD^+ - nicotinamide-adenine dinucleotide, oxidized form

NIH - National Institutes of Health

ODC - ornithine decarboxylase

ODCase - orotidine-5'-decarboxylase

OPRT - orotate phosphoribosyltransferase

PAGE - polyacrylamide gel electrophoresis

PAS - (para-aminosalicylic acid)

pBAce - pB = Bluescript® phagemid, Ace = alkaline phosphatase promoter regulated expression

PCR - polymerase chain reaction

PKDL - post kala azar dermal leishmaniasis

PPD - purified protein derivative

PRPP -(5-phosphoribosyl, 1-pyrophosphate)

PZA - pyrazinamide

RNA - ribonucleic acid

SAMDC - S-adenosylmethionine decarboxylase

SDA-FP - strand displacement amplification / fluorescence polarization

SDS - soduim dodecyl sulfate

SOD - superoxide dismutase

SSCP - single strand conformation polymorphism

TB - tubewrculosis (the disease)

TE - Tris, EDTA (edetic acid)

TMA -transcription mediated amplification

TNF-α - tumor necrosis factor

TR - trypanothione reductase

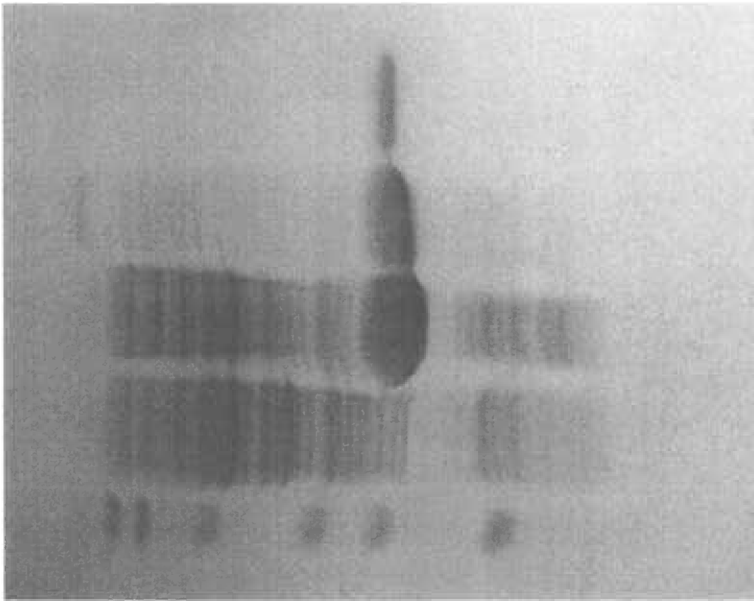
XPRT - xanthine phosphoribosyltransferase

Figure 40. Purification of HGPRT.

M. avium and *M. tuberculosis* HGPRT was purified by GTP-agarose affinity chromatography.

Figure 41. Purification of *M. tuberculosis* HGPRT.

M. tuberculosis HGPRT was purified using differential ammonium sulfate precipitation followed by size separation on a G-100 gel permeation column.



M. t. HGPRT purified

25% $(NH_4)_2\ SO_4$ ppt.

M.t. HGPRT induction

pBAce Control

STD