A Genetic Analysis of the Purine Salvage Pathway of *Leishmania donovani*

by

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Summary of Thesis Work

Billions of people are infected with parasites, the causative agents of a wide variety of devastating and often fatal diseases. Leishmaniasis, caused by species of the genus *Leishmania*, is a parasitic disease that presently affects ~12 million people in 88 countries, with an additional estimated 350 million at risk for infection. No anti-leishmanial vaccine exists that can prevent leishmanial infections, and current drug regimens are toxic and ineffective in > 40% of the patients with the visceral, fatal form of the disease. The need for better and more efficacious drugs for the treatment of leishmaniasis—and for that matter of any parasitic disease—is acute. The development of a rational, specific, and effective anti-parasitic drug therapy depends upon the exploitation of any unique, fundamental discrepancy between parasite and host.

Perhaps the most striking difference between parasites and their mammalian hosts is the pathways by which the two synthesize purine nucleotides. All protozoan parasites studied to date are incapable of synthesizing purine nucleotides *de novo* and therefore must rely upon purine salvage to obtain obligatory purines from their hosts. Therapeutic exploitation of such fundamental biochemical differences between pathogen and host offers a selective rational treatment for these and other parasitic diseases.

Leishmania express four enzymes capable of salvaging preformed purines to the nucleotide level: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), adenine

phosphoribosyltransferase (APRT), and adenosine kinase (AK). To establish the relative contributions of HGPRT, APRT and AK to purine salvage, *L. donovani* mutants deficient in these enzymes were generated in every conceivable combination using chemical mutagenesis and gene replacement approaches. All mutants, including, the *ak⁻/Δhgprt/Δaprt* triple mutant, were viable and could salvage all naturally occurring purine bases and nucleosides. We therefore concluded that parasites deficient in APRT, HGPRT and AK divert purine salvage through XPRT.

HGPRT is known to be in the glycosome, a fuel-metabolizing microbody found in *Leishmania* and trypanosomes, and XPRT was hypothesized to be in the same location because it contained a putative glycosomal targeting signal. We hypothesized that perhaps the reason the parasites needed either HGPRT or XPRT (see below) was because they were both in the glycosome. Therefore, the first aim of my thesis project was to localize the XPRT protein. Once I had confirmed that XPRT was located in the glycosome, it was necessary to determine if its location was necessary for its function. This entailed deleting the C-terminal tri-peptide glycosomal targeting signal from the protein and expressing the mutant xprt within a *Δxprt* null background. The xprt protein was localized to the cytosol, and biochemical and growth analysis were performed on the parasites with the mis-localized protein. It was concluded that the function of XPRT does not depend on its location.

The second aim of my thesis project was to evaluate whether a *Δxprt* mutation could be introduced into wild type *L. donovani* and all of the purine salvage mutants mentioned above. I generated the *XPRT/xprt* heterozygous mutation in wild type cells and in each of the mutant cell lines and successfully inserted the homozygous *Δxprt* mutation into wild type and *ak⁻/Δaprt* cells; however, *Δxprt* could not be inserted into any cell line that contained the *Δhgprt* mutation. The ability to create an *ak⁻/Δaprt/Δxprt* mutant, along with the existence of the previously generated *ak⁻/Δhgprt/Δaprt* mutant, established that *L. donovani* promastigotes could survive with either HGPRT or XPRT alone. These genetic studies implied, but did not prove, the central hypothesis of my Ph.D. thesis research: that either HGPRT or XPRT is both necessary and sufficient for the viability and growth of *L. donovani*. To test this hypothesis, I attempted to generate a *Δhgprt/Δxprt* double knockout in the presence of a cocktail of exogenous purines (to circumvent a potentially lethal genetic block) using a multitude of schemes involving targeted gene replacement, episomal complementation, and chemical selection. Although it was possible to generate a *Δhgprt/XPRT/xprt* mutant, all efforts to generate the *Δhgprt/Δxprt* double knockout using these techniques were unsuccessful. These negative results still did not prove my central hypothesis. Therefore, confirming the hypothesis that either HGPRT or XPRT is necessary and sufficient for the growth and survival of *L. donovani* promastigotes, or disproving it by generating a *Δhgprt/Δxprt* mutant became the third goal of my thesis project.

Several reports in the literature have implied that all exogenous purines are funneled to hypoxanthine and xanthine in the parasite before conversion to the nucleotide level. If true, this afforded a logical explanation for why the *Δhgprt /Δxprt* mutation would be lethal, since there would be no route for purine incorporation into the nucleotide pool of a *Δhgprt /Δxprt* double knockout. Because *L. donovani* promastigotes express an adenine aminohydrolase (AAH, LdAAH) theoretically capable of converting 6-aminopurines to hypoxanthine, I added an inhibitor of LdAAH, 2'-deoxycoformycin (dCF), to the purine cocktail in the last round of transfection. The theoretical basis for using dCF in the selection medium was to prevent adenine deamination and enable a *Δhgprt/Δxprt* to salvage adenine via APRT. The strategy worked, and a viable *Δhgprt/Δxprt* null mutant was isolated in the presence of dCF.

Unlike wild type promastigotes, the *Δhgprt/Δxprt* null mutant is absolutely dependent upon the presence of dCF and either adenine or adenosine as a purine source. The *Δhgprt/Δxprt* mutant cannot grow on guanine, guanosine, hypoxanthine, xanthine or xanthosine, but surprisingly can grow, albeit meagerly, on inosine. These data establish genetically that either HGPRT or XPRT is essential for purine acquisition and parasite viability and that all exogenous purines are funneled to hypoxanthine and/or xanthine by *L. donovani* promastigotes.

The final goal of my thesis project was to examine the purine salvage pathway of *L. donovani* amastigotes and determine if HGPRT, APRT, XPRT, or

combinations thereof, is necessary for transformation of promastigotes into their infective amastigote life stage. I generated *Δhgprt*, *Δaprt*, *Δxprt* and *Δhgprt/Δxprt* knockouts within a virulent wild type strain of *L. donovani,* LdBob, that is capable of axenic transformation into axenic amastigotes. The *Δaprt*, *Δhgprt* and *Δxprt*, as well as the *Δhgprt/Δxprt*, strains were all capable of transforming into axenic amastigotes and proliferating indefinitely as long as appropriate purines were provided to sustain growth. The nutritional requirements of the amastigotes were essentially the same as those of their promastigote counterparts. Additionally, wild type, *Δaprt*, *Δhgprt* and *Δxprt* parasites could all infect and proliferate within bone-marrow derived macrophages *in vitro*. However, the *Δhgprt/Δxprt* mutants could not replicate within macrophages, even though their initial infection rate is equivalent to that of the wild type strain. The inability of the *Δhgprt/Δxprt* mutants to proliferate within macrophages suggests they could be exploited as a live vaccine against leishmaniasis. *In vivo* studies in hamsters will soon be initiated in order to determine what level of infection the *Δhgprt/Δxprt* mutants are capable of causing in a rodent model of the disease.

In summary, my findings establish genetically that: 1) XPRT is located in the glycosome, and that a glycosomal location is not required for function 2.) either HGPRT or XPRT is absolutely essential for purine acquisition, parasite viability, and parasite infectivity of mouse macrophages, and 3) all exogenous purines are funneled to hypoxanthine and/or xanthine in promastigotes. I have proven my central hypothesis and demonstrated the existence of many activities

within the functionally redundant purine salvage pathway whose activities had been only hypothesized or alluded to indirectly. Overall, the knowledge gained from my Ph.D. thesis investigations offers an avenue both for the prevention of leishmaniasis through a live vaccine strategy and for the treatment of the disease either by the discovery or development of an inhibitor that targets the two central enzymes of purine salvage in the parasite, or through targeting the highly active LdAAH enzyme via a subversive substrate..

Chapter 1

INTRODUCTION and BACKGROUND

1.1. Parasites

A parasite is an organism that grows, feeds, and is sheltered on or in a different organism while contributing nothing beneficial to its host (1). Billions of people around the world are infected with parasites, the causative agents of a wide variety of devastating and often fatal diseases. Parasitic infections are a major health problem in the developing world, where there is significant overpopulation and, as a consequence of limited resources and political instability, provision of health care is either substandard or nonexistent.

Parasitic diseases are caused by a variety of organisms belonging to the classes of Trematoda and Cestoidea, the phylum Nematoda, as well as the subkingdom of Protozoa (2). The classes of Trematoda and Cestoidea belong to the phylum platyhelminthes and include flatworms that are parasitic to humans. Trematoda includes liver flukes, lung flukes, intestinal flukes and species of the genus *Schistosoma*, members of which are responsible for an excess of 250

million cases of human schistosomiasis (2). The class Cestoidea comprises intestinal tapeworms, including the fish and pork tapeworms, *Diphyllobothrium latum* and *Taenia solium*, respectively, as well as some species of extraintestinal tapeworms. Diseases caused by non-segmented roundworms that belong to the phylum Nematoda are a major contributor of human parasitic diseases, and are often more serious than those caused by trematodes and cestodes. For example, *Strongyloides stercoralis*, which can cause a fatal disease in children known as swollen belly syndrome, is estimated to infect 100-200 million people worldwide (2). *Wuchereria bancrofti*, which infects more than 120 million people worldwide, is parasitic only in humans. *W. bancrofti* causes a lymphatic disease known as Bancroft's filariasis, which is characterized by severe enlargement of the extremities, and is known more commonly by the misnomer elephantiasis (2).

The subkingdom of Protozoa consists of approximately 66,000 different species and encompasses a great amount of human parasitic disease burden throughout the world. Millions protozoan infections in humans are caused by four genera of parasitic protozoa: *Plasmodium, Trypanosoma cruzi, Trypanosoma brucei* and *Leishmania*. Together, these parasites infect about 500 million people worldwide, and cause millions of deaths each year, with billions of people at risk for infection (3). Malaria, caused by *Plasmodium falciparum*, is estimated to infect an additional 250-300 million people each year, with more than 1 million deaths occurring annually. Due to its astronomically high disease burden and the high number of deaths it causes each year, malaria remains one of the greatest

killers of all time (3). Other common diseases caused by protozoan parasites include amoebiasis, babesiosis, giardial enteritis, cryptosporidiosis, ocular keratitis, keratoconjuntivitis, primary amoebic meningoencephalitis, urethritis, vaginitis and toxoplasmosis (2).

Chemotherapeutic intervention is one of the main treatments of parasitic diseases. However, most of these agents are far from ideal. Many are moderately to highly toxic to humans and often require prolonged therapy with multiple drug administrations by trained personnel (3). In addition, parasite resistance to present chemotherapies continues to be an ongoing challenge to disease treatment; therefore new and improved antiparasitic agents are essential. As a step toward this goal, the focus of this doctoral thesis has been a genetic dissection of the purine salvage pathway of *Leishmania donovani*, the causative agent of visceral leishmaniasis. I have put forth an effort to validate the metabolic functions of the proteins within the purine salvage pathway of *Leishmania donovani*, with my ultimate goals being to evaluate these enzymes as potential targets for antiparasitic chemotherapy and to develop a strain of *L. donovani* that is a potential leishmaniasis vaccine candidate.

1.2. Classification and Unique Features

Leishmania species are unicellular protozoan parasites of the Kinetoplastid order and Trypanosomatidae family. Also included in this family are *Crithidia*, *Blastocrithidia*, *Endotrypanum*, *Herpetomonas*, *Leptomonas*,

Phytomonas and the *Wallacenia* species *Trypanosoma brucei* and *Trypanosoma cruzi*. The latter two are the causative agents of African sleeping sickness and Chaga's disease, respectively (4, 5). Species of the order Kinetoplastida contain a number of unique attributes that will briefly be discussed here. Perhaps the most unique structural feature of parasites of this order is the kinetoplast, an organelle rich in DNA that is contained within the single mitochondrion of a kinetoplastid cell (2, 4).

The kinetoplast (whose name means "motion body") was originally thought to be involved in the motility of *Leishmania* and trypanosomes (4). It typically lies posterior to the flagellar base of kinetoplastids, and contains 10-20% of the total cellular DNA (Fig. 1) (4, 6). The kinetoplast is composed of a limited number of nucleotides organized as two classes of circular DNAs, mini-circles and maxi-circles, which are catenated into a single network within each cell. There are approximately 50 copies of maxi-circle DNA in each kinetoplast, and these encode rRNA genes and mitochondrial genes such as cytochrome b, cytochrome b oxidase and NADH hydrogenase (4, 7). In contrast, there are approximately 5,000 copies of mini-circles per parasite, and these encode small guide RNAs that are used to edit messenger transcripts, which are then transcribed as encrypted mRNAs, a process known as RNA editing. RNA editing and post-transcriptional alteration of kinetoplast-encoded sequence occurs within the mitochondria before translation of the final protein. Thus, the final mRNA transcript produced combines RNA transcribed from the mini-circles of the

kinetoplast and portions transcribed from the circular mitochondrial genome (4). Kinetoplastidae also use trans-splicing, instead of cis-splicing, in the processing of nuclear-encoded messenger RNA. The messenger RNAs are transcribed in an unusual polycystronic manner and spliced into individual messenger RNAs by a reasonably well characterized splicing machinery. Trans splicing involves the addition of a specific 39 nucleotide RNA, designated the mini-exon, or splice-leader sequence, that is appended onto the 5' end of the original transcripts (8).

Another unique characteristic of *Leishmania* and other members of the Trypanosomatidae family is the existence of a specific fuel-metabolizing microbody called the glycosome (9). Glycosomes are similar to peroxisomes, organelles that function to remove toxic substances from cells, with the exception that they lack catalase, a hallmark enzyme of peroxisomes (9). Glycosomes compartmentalize enzymes involved in glycolysis, ether-lipid and sterol synthesis, the fatty acid and β-oxidation pathway, pyrimidine biosynthesis, and purine salvage (10). It is not certain why it is necessary for the parasites to concentrate activities within the glycosome; however, the occurrence of some evolutionary event that made compartmentalization of certain proteins advantageous to *Leishmania* and other trypanosomes has been hypothesized (11). It has also been hypothesized that sequestration enables high levels of enzymes to be concentrated in one place, therefore resulting in a high glycolytic flux. Other ideas suggest that the glycosome is a compartment in which ATP

concentration can rapidly increase, while keeping the ATP/ADP balance under control so as not to cause osmotic rupture of the cell.

The most striking biochemical feature of the Kinetoplastidae is their inability to synthesize the purine ring *de novo*. These parasites have developed an intricate purine salvage pathway that enables them to obtain host purines. Evaluating the purine salvage pathway of *Leishmania donovani* by genetic means, as well as the localization of an additional purine salvage enzyme within the glycosome, is an important part of this thesis. Another unique biochemical feature that has been found in all trypanosomatids, but in no other organisms examined to date, is the requirement of an unusual low molecular weight cofactor for the activity of glutathione reductase. This compound, N^1,N^8-bis(ι-γ-glutamyl-ι-hemicystinyl-glycyl)spermidine, has been given the name trypanothione (12). Trypanothione plays a pivotal role in a number of parasite processes, such as regulation of intracellular thiol redox balance, synthesis of deoxyribonucleotides, drug resistance, and defense against chemical and oxidant stress (13). In *Leishmania* it has also been implicated in the mode of action of antimonials and in resistance to trivalent antimony in laboratory-derived resistant strains (13).

1.3. *Leishmania* Parasites

1.3.1. Discovery of *Leishmania*

Leishmania was co-discovered in 1903 by William Leishman, a Glaswegian doctor serving with the British army in India (14) and Charles Donovan, a British doctor working in Madras, India (15). Leishman was the first to publish observations of ovoid bodies that he found in the spleen of a British soldier who had died of visceral leishmaniasis, and Donovan published similar results shortly thereafter. The "ovoid bodies", now known as amastigotes, were termed "Leishman-Donovan bodies", and the species of invading parasite was named *Leishmania donovani* (14).

1.3.2. *Leishmania* life cycle

Leishmania are digenetic protozoa, existing in both mammalian host and insect vector (Fig. 2). The ovoid amastigotes develop intracellularly within the vertibrate host and are characterized by a single, prominent nucleus and a very short or absent flagellum. The unique capability of intracellular *Leishmania spp.* amastigotes to adapt to life within macrophages allows them to evade host immune systems and reside comfortably within host cells, ultimately resulting in disease. Extracellular promastigotes proliferate exclusively in the insect vector. They are elongated and contain a long, anterior, free flagellum which serves for locomotion and enables the parasites to attach to the wall of the insect gut (2). They mature in the midgut of the sand fly and are inoculated into mammalian host tissue during a blood meal. The promastigotes are readily phagocytosed by mammalian macrophages wherein they transform into amastigotes (16, 17).

1.3.3. Host-parasite interactions

Leishmania amastigotes are taken up, mostly within infected macrophages, during a blood meal and held within the sandfly midgut by a peritrophic matrix. Parasites are released from macrophages and differentiate into dividing procyclic promastigotes. After a few days, the peritrophic matrix breaks down, and parasites attach to the luminal surface of the sandfly midgut via an abundant parasite surface glycolipid: lipophosphoglycan (LPG). LPG which consists of a modified phosphatidyl inositol lipid anchor, a glycan core, repeating disaccharide phosphate units and a small oligosaccharide cap (18). The parasites replicate extensively while attached to the sandfly midgut. As the blood meal is digested, replication ceases, and the parasites differentiate into metacyclic promastigotes. Changes in the LPG structure reduce the ability of these highly infectious metacyclics to bind the sandfly midgut. This releases the parasites from the midgut, enabling them to migrate elsewhere—particularly into the alimentary tract of the sandfly—in preparation for transmission to a new mammalian host during the sandfly's next blood meal (19). Upon inoculation, the metacyclics must first resist the action of a serum complement, then bind and enter macrophages where they differentiate into the amastigote stage. As amastigotes mature, LPG synthesis is shut down and the parasites adapt for life within the fusogenic, acidified phagolysosome of the macrophage. Whether amastigotes are released by the bursting of host cells, or by mediated exocytosis

where macrophages eventually succumb to apoptosis or necrosis, is not clear (20).

1.4. Disease manifestation and occurrence

1.4.1. Leishmaniasis

No thesis on leishmaniasis should trivialize the havoc that leishmaniasis causes humans in terms of mortality and economic spoliation. There are at least three well-characterized forms of leishmaniasis—cutaneous, mucocutaneous, and visceral—each caused by different species and subspecies of *Leishmania*. More than 12 million people in 88 countries, including the United States and parts of Europe, are presently afflicted with leishmaniasis, and an estimated 350 million people are at risk for infection. There are ~ 2 million new cases of leishmaniasis each year, and ~ 60,000 people die annually from this disease (3). American soldiers and Allied forces who are currently in areas of the Middle East, such as central Iraq, the Greater Baghdad area and Afghanistan, are at high risk for infection with cutaneous and visceral leishmaniasis (3). In fact, the number of cases of cutaneous leishmaniasis occurring in U.S. soldiers has increased from 22 in 2002-2003 (21) to 830 reported cases since the U.S invasion of Iraq in March 2003, and may be as high as 1500 (22). In addition, 4 U.S. soldiers have also been infected with visceral leishmaniasis, the fatal form of the disease (22). As more soldiers are deployed to these areas, we are likely to see a marked

increase in the number of cases of leishmaniasis among our troops. Currently, no vaccine exists that can prevent leishmanial infections, and current drug treatments are expensive, difficult to administer and ineffective in up to 40% of the patients with visceral leishmaniasis. Moreover, many of the anti-leishmanial chemotherapies have a non-specific mechanism of action, and treatments with these drugs may cause irreversible toxic damage, such as blindness, diabetes or even death. In addition, the evolution of drug-resistant parasites is on the rise (3), which makes it even more difficult to develop treatments that can endure parasite defense mechanisms.

Leishmaniasis can manifest in one of three forms: visceral, cutaneous, or mucocutaneous. Different species of the genus *Leishmania* cause different forms of the disease (Table 1), and each form of the disease carries with it a broad spectrum of symptoms. Approximately 21 species of *Leishmania* are transmitted by over 30 different species of sandfly vectors, and specific species of both parasite and vector reside in different parts of the world (18), (Fig. 3). It is widely believed that the geographical distribution of the vector determines which species of *Leishmania* is endemic to a particular area.

1.4.2. Visceral leishmaniasis

L. donovani, *L. infantum* and *L. chagasi* cause visceral leishmaniasis, the most harmful form of the disease. More than 90% of visceral leishmaniasis cases occur in Bangladesh, Brazil, India and Sudan (3). *L. donovani* is found in

the Mediterranean and Southern Europe, North and Eastern Africa, India and China, while *L. infantum* resides in the Mediterranean basin and Southwest Asia. Both of these species are transmitted by the *Phlebotomus* genus of sandfly. *L. chagasi* is found in Brazil and is transmitted by the *Lutzomyia* genus of sandfly. (18).

Visceral leishmaniasis symptoms range from an asymptomatic or mild infection to a severe-life-threatening disease known, depending on one's location, as Dum-dum fever, Assam fever, or most commonly, Kala-azar (18, 23). The name "kala azar" directly translates into "black fever", and it is named for the darkening of skin on the hands, face and abdomen, that accompany a visceral infection. Visceral-disease causing parasites reside within the reticuloendothelial cells of the viscera, including the spleen, lymph nodes, liver and intestine (18). Visceral leishmaniasis has an incubation period of 2-4 months, and if left untreated, swelling of the liver and spleen, and death are inevitable (18, 23).

1.4.3. Cutaneous leishmaniasis

Cutaneous leishmaniasis has an incubation period of a few days to months and is characterized by skin lesions that may self-heal over time. The lesions generally remain localized to the site of the sandfly bite. 90% of cutaneous leishmaniasis cases occur in Afghanistan, Iran, Nepal, Syria, Saudi Arabia, Brazil and Peru (3). Species of *Leishmania* that cause cutaneous

leishmaniasis include, but are not limited to, *L. mexicana*, *L. amazonensis, L. aethiopica* and *L. panamensis (3). L. mexicana* is also found in Central and North America, including Mexico and Texas. A cutaneous leishmaniasis infection acquired from *L. mexicana* may also affect the mucocutaneous regions of the body; and an infection from *L. amazonensis* or *L. aethiopica* can cause diffuse leishmaniasis that spreads over the entire body (18).

L. major and *L. tropica* can both cause a form of cutaneous leishmaniasis, termed dermal leishmaniasis, in which the amastigotes replicate within the reticuloendothelial system and lymphatic cells rather than staying in the vicinity of the sandfly bite. *L. major* is found in sparsely inhabited areas of North Africa, the Middle East, West India and Sudan. It cycles mainly through gerbils and other small rodents, with humans as an incidental host. *L. tropica* is found in Ethiopia, Kenya, North Africa, the Middle East, the European Mediterranean and India, wherein it is confined mostly to densely populated areas. It is transmitted between humans, with dogs serving as a reservoir host (18).

1.4.4. Mucocutaneous leishmaniasis

The third type of leishmaniasis, mucocutaneous leishmaniasis, mutilates or entirely destroys the mucous membranes within the nose, mouth and throat. Designs on pre-Columbian pottery (Fig. 4) and the existence of thousand-year-old skulls with evidence of mucocutaneous leishmaniasis prove that this form of the disease has existed in the Americas for a long time (3, 23). Mucocutaneos

leishmaniasis is caused by *L. braziliensis*, which is found in Central and South America, and is spread by the *Lutzomyia* species of sandfly. Primary lesions occur at the site of the bite, but infection also involves the mucosal system of the nasal and buccal cavity. There, cartilaginous and soft tissues including the lips, nose, hard and soft palates and vocal cords, ulcerate and degenerate, resulting in horrific disfigurement. Death from mucocutaneous leishmaniasis usually occurs from a secondary infection or malnutrition (18).

1.4.5. Leishmaniasis-HIV Co-infection

Concerns regarding leishmaniasis-human immunodeficiency virus (HIV) co-infection are growing, particularly in Southern European countries, where up to 70% of all adult cases of visceral leishmaniasis are associated with HIV/acquired immunodeficiency syndrome (AIDS) and up to 9% of all AIDS cases suffer from newly acquired or reactivated visceral leishmaniasis (24). Leishmaniasis-HIV co-infection is considered an emerging disease, particularly in urban areas where intravenous drug usage can promote the spread of HIV. Although co-infection of HIV with visceral leishmaniasis is more prevalent, there have also been cases of HIV-cutaneous leishmaniasis co-infection (24).

Both HIV and *Leishmania* parasites can infect and multiply within macrophages as well as interfere with the immune system, and infection with one increases the chances of infection with the other (24). Leishmaniasis can activate HIV in latently infected monolytic and T-cells. It may also decrease HIV-

specific cytotoxic T-lymphocyte (CD8) response, or it may depress activity of first stage immune response cells (T_h1) and enhance the activity of the second-stage immune response cells (T_h2) (24). Conversely, HIV can provide favorable conditions for a *Leishmania* infection by deactivating macrophage function, depressing the activity of T_h1 cells, inhibiting phagolysosome fusion, and also by enhancing the activity of T_h2 cells (24).

1.5. Treatments, drug resistance and drug development

1.5.1. Chemotherapy

Some forms of leishmaniasis respond well to treatment with pentavalent antimonials which were first enlisted as an anti-leishmaniasis chemotherapy in 1945 (25). These compounds are usually administered intravenously for up to 28 days, and treatment often fails. The mechanism of action of the antimonials against *Leishmania* is poorly understood. It is hypothesized that antimony may inhibit trypanothione production, ADP phosphorylation, DNA topoisomerase I, or fatty acid β-oxidation (25). Other anti-leishmanial compounds, including those that are effective against antimony-resistant parasites, have also been developed. However, neither the antimonials, other anti-leishmanial compounds, nor combinations thereof have been able to cure 100% of the patients infected with any form of leishmaniasis as tested to date. In addition, strain variation in

sensitivity and increasing parasite resistance to these compounds, as well as their potential patient toxicity and the excessive amount and invasive way in which the drugs must be administered, makes them less than ideal for continued use in combating leishmaniasis (3, 25, 26).

The leading antileishmanial drugs currently recommended for chemotherapeutic treatment of visceral leishmaniasis include two pentavalent antimonials; sodium stibogluconate (Pentostam™, GlaxoSmithKline; http://www.gsk.com), and meglamine antimoniate (Glucantime, Aventis; http://www.aventis.com), as well as Pentamidine (Aventis), amphotericinB (Bristol-Myers Squib; http://www.bms.com) and its lipid formulation AmBiosome® (Gilead; http://www. gilead.com) (25, 26). Other compounds and formulations that have recently been tested for use against *Leishmania* parasites include Miltefosine, Paromomycin and Aladara (25). The mechanisms of action of most of these drugs are summarized in Table 2, and their structures shown in Figure 5.

The toxic effects they exhibit in humans often limit the usefulness of some of these anti-leishmanial compounds. For example, pentamidine, which is usually used as a second line treatment for antimony-resistant cases of visceral leishmaniasis, can cause hypoglycemia and nephrotoxicity. Miltefosine has been hailed as a novel oral drug for the treatment of visceral leishmaniasis, but its teratogenicity excludes its use in women of childbearing age (25). Therefore, even though Miltefosine has had a high rate of success in the treatment and cure of antimony-resistant cases of visceral leishmaniasis in India and cutaneous

leishmaniasis in Columbia, its usefulness as a first-line anti-leishmanial compound is diminished. Not all anti-leishmanial compounds are as toxic as the pentavalent antimonials or Miltefosine. AmBiosome®, the lipid-associated formulation of amphotericin B, is less toxic than its parent compound. Trials to test the efficacy of single-dose AmBiosome® as a treatment for visceral leishmaniasis are underway and report a 90% cure rate to date, however its high monetary cost has limited its use in patients (25, 26). Paromomycin, an aminoglycoside antibiotic, has shown promise for use as a topical treatment for cutaneous leishmaniasis, but its lack of penetration to subcutaneous levels renders it a poor treatment for the dermal form of leishmaniasis that can be caused by *L. major* and *L. tropica* (25, 26). Paromomycin is therefore usually used in combination with antimonials (25). Another topical cream, Aladara™, (3M Pharmaceuticals; http://www.3m.com) induces nitric oxide (NO) production in macrophages *in vitro* killing intracellular *L. donovani* amastigotes (26) (25). The active ingredient in Aladara™, imiquimod, has been used in humans for the treatment of other diseases, which makes it a promising agent for use in leishmaniasis therapy (25).

1.5.2. Drug resistance in *Leishmania*

Leishmania have developed resistance to many of the chemotherapies currently being used to treat the various forms of leishmaniasis. Drug resistance can be defined as a decline in the efficacy of a drug against a population

previously susceptible to a compound. This definition assumes that the original susceptibility of the population is known, which is not always the case for *Leishmania*. Indeed, leishmanial resistance against a given drug may be natural, or may be acquired when the parasites are exposed to sub-optimal drug doses (27). Rather than succumb to chemotherapy, the *Leishmania* parasites can protect themselves through a variety of genetic modifications, mutation, inactivation, and/or gene amplification, which occur in response to the drugs administered and induce parasite resistance to many of the anti-leishmanial compounds used to date. Gene amplification appears to be quite common in *Leishmania* whereas it is rare in *Trypanosomes* (19). Resistant phenotypes selected *in vitro* are usually obtained by culturing wild-type parasites under increasing drug concentrations. The wild-type parasites may be either heterogeneous (non-cloned) parasites or isolated cloned colonies with high genetic homogeneity. For example, antimony-resistant *L. tarentolae* that were selected for their resistance *in vitro* exhibit an increased synthesis of intracellular thiols, including an up to 40-fold increase trypanothione (28). A model for drug resistance involving trypanothione is proposed in which Sb(V) containing compounds, such as Pentostam, are reduced intracellularly to Sb(III), conjugated to trypanothione and extruded from the cell (28).

Pentamidine and other diamidines are thought to accumulate in the *Leishmania* mitochondrion. Pentamidine-resistant *L. mexicana* have demonstrated a lack of intramitochondrial pentamidine accumulation and

increased cellular extrusion of the drug (27, 29, 30). Alterations in the membrane fluidity, lipid content and loss of pentamidine binding sites in *L. amazonensis* have also been described (27). Amphotericin B binds preferentially to ergosterol, the major sterol of fungi, *Leishmania* and *Trypanosoma* cruzi. Amphotericin B resistant *L. donovani* promastigotes selected *in vitro* for resistance to amphotericin B exhibited an increase in membrane fluidity and a major change in membrane composition, including replacement of ergosterol with an ergosterol precursor, cholesta-5,7,24-trien-3β-ol (31), which enables the parasites to decrease influx and increase efflux of the drug (31).

1.5.3. Other limitations to drug development

Developing drugs to treat leishmaniasis has been a difficult process, and the therapies produced to date are not wholly effective. Aside from parasite-resistance to chemotherapeutic agents, other limitations to anti-leishmanial drug developments are cost and interest (13, 25, 32). Many compounds are capable of inhibiting specific protein targets in laboratory studies, or exhibit toxicity against *Leishmania* with an unknown mode of action, but translation of these studies into development of drugs with higher efficacy and testing of such drugs in clinical trials is difficult. The drug development process is expensive and time-consuming. Funding is available for basic research, but the lack of successful drug development is related to the lack of an appropriately sized market for treatment of parasitic diseases, such as leishmaniasis and trypanosomiasis (32).

In other words, the development of drugs to treat those affected in the tropical and subtropical regions of the world is not a profitable business for pharmaceutical companies; therefore, interest is decreased.

If a 100% effective anti-leishmanial compound is to be discovered, or an anti-leishmaniasis vaccine is to be created, a thorough understanding of parasite biology and metabolic pathways is a prerequisite for this goal. Continued research is necessary to determine what types of genes may be "essential genes", whose protein products might make good drug targets, or to determine whether there are gene deletions that result in discontinued production of a vital protein product that may render a strain of *Leishmania* practicable for use as an attenuated vaccine. It is also necessary to understand in more detail the metabolic pathways and functions within *Leishmania* parasites and determine which of these may differ from those within mammalian hosts. However, more fundamental research is essential to gain the requisite knowledge to rationally exploit these differences and effectively treat or prevent leishmaniasis.

1.6. Purine biosynthesis and salvage

1.6.1. Functions of purines

The purine salvage pathway of *Leishmania donovani* is the focus of my thesis. Purine nucleotides play myriad functions in living cells. They serve as precursors for a variety of products, including ATP, the universal currency of

cellular energy; cyclic AMP and cyclic GMP as second messenger molecules in cell signaling pathways; and nucleotides as constituents of activated intermediates involved in many biosynthetic pathways. Purine nucleotide triphosphates are also the precursors of DNA and adenine nucleotides are component parts of NAD(P+), FAD, and CoA, each a vitamin-derived coenzyme required for many of the key enzymes of intermediary metabolism (33).

1.6.2. Structure of Purines

Naturally occurring purines exist as nucleobases, nucleosides and nucleotides; although the structure of various purines is biochemistry textbook material, distinctions among the structures of these purines played a critical role in this thesis work. Therefore, their structures are briefly described here. Purine nucleobases consist of two heterocyclic rings of carbon and nitrogen with the general formula C_5N_4 (Fig. 6A). The purine nucleobases relevant to this thesis include adenine, hypoxanthine, xanthine and guanine. The structure of adenine is shown in (Fig. 6B). A purine nucleoside is a purine nucleobase in which the 9-nitrogen is covalently attached to the 1-carbon of a ribose or deoxyribose sugar in an N-glycosidic linkage. Nucleosides relevant to my thesis work include adenosine, guanosine, inosine and xanthosine. The structure of adenosine is also shown (Fig. 6C). A purine nucleotide is a purine nucleoside in which 1, 2 or 3 phosphate groups are attached to the 5'-hydroxyl group of the sugar group that is attached to the nucleobase (Fig. 6D).

1.6.3. Purine biosynthesis

In mammalian cells, purine nucleotides are generated by two pathways: a biosynthetic pathway wherein purine nucleotides are synthesized from amino acids and one-carbon moieties, and a salvage pathway in which purine nucleotides are synthesized from pre-formed bases and nucleosides. The *de novo* biosynthetic pathway is conserved in all living organisms capable of synthesizing the purine ring, and involves the assembly of this purine heterocyclic ring onto a ribose 5-phospate backbone. The purine ring is assembled from a variety of precursors: glutamine, glycine, aspartate, methylenetetrahydrofolaate, N^{10}-formyltetrahydrofolate, and CO_2. There are eleven steps in *de novo* purine biosynthesis. Briefly, it begins with the synthesis of 5-phospho-α-D-ribose-1-diphosphate (PRPP). PRPP is synthesized from ATP and ribose 5-phosphate, the latter of which is a bi-product of the hexose monophosphate shunt, in a reaction catalyzed by PRPP synthetase. Next, 5-phosphoribosylamine is formed from PRPP and glutamine. The addition of glycine, followed by formylation, amination and ring closure yields 5-aminoimidazole ribonucleotide, an intermediate that contains the completed five-membered ring of the purine skeleton (Fig. 6A). The addition of CO_2, the nitrogen atom of aspartate, and a formyl group, followed by ring closure, yields inosinate (IMP), a purine ribonucleotide that can be converted to adenosine monophosphate (AMP) or guanosine monophosphate (GMP). AMP is formed from IMP by the addition of

ammonium succinate by adenylosuccinate synthetase (ADSS), then the succinyl group is removed by adenylosuccinate lyase (ASL) to form AMP. IMP is converted to xanthosine monophosphate (XMP) via the dehydrogenation action of IMP dehydrogenase (IMPDH), and the XMP is subsequently aminated to GMP by GMP synthase (GMPS). Once AMP and GMP are synthesized, they are converted to diphosphates by specific nucleoside monophosphate and nulcleoside diphosphate kinases in which ATP serves as the phosphate donor.

Regulation of key enzymes ensures a balanced supply of adenylate and guanylate nucleotides. These include PRPP synthetase and PRPP-glutamine amidotransferase, each of which is subject to feedback inhibition by nucleotides. Moreover, branch-point enzymes, such as ADSS and IMPDH, are regulated in order to ensure a balanced supply of adenylate and guanylate nucleotides. ADSS is inhibited by AMP and requires GTP. IMPDH is inhibited by GMP and GMP synthetase requires ATP (Fig. 7A).

Purine nucleotides are continuously catabolized, ultimately producing uric acid. Specifically, AMP is deaminated to IMP by adenylate deaminase, and IMP is catabolized to inosine, via a nucleotidase. The 1-ribose-phosphate group is removed from inosine by purine nucleoside phosphorylase to form hypoxathine, and hypoxanthine is oxidized to xanthine by xanthine oxidase, which can subsequently degrade xanthine to uric acid and then to urate. GMP is taken down to guanine by purine nucleoside phosphorylase, and guanine is

deaminated to xanthine, then oxidized to uric acid via guanine deaminase and xanthine oxidase, respectively (Fig. 7B).

1.6.4. Purine salvage in humans

Purine ribonucleotides can also be synthesized through a salvage pathway in which the base released from nucleic acid and nucleotide recycling is directly phosphoribosylated with the phosphoribosyl group from PRPP. Humans possess two phosphoribosyltransferases (PRTs), enzymes that transfer the sugar-phosphate group from PRPP to the nucleotide base: Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) phosphoribosylates hypoxanthine and guanine to IMP and GMP, respectively, and adenine phosphoribosyltransferase (APRT) phosphoribosylates adenine to form AMP. In addition, they also have an adenosine kinase (AK) that directly phosphorylates adenosine using ATP as the phosphate donor.

1.6.5. Purine salvage deficiencies in humans

A partial deficiency in HGPRT increases the degradation of guanine and hypoxanthine and results in an accumulation of urate. The excessive amount of urate produced from this process can precipitate as sodium urate crystals within the joints. This can cause extremely painful joint swelling and a disease known as gout. Allopurinol, a hypoxanthine isomer, is the favored treatment for gout. Allopurinol first acts as a substrate of xanthine oxidase, then the product of the

oxidation reaction, alloxanthine, serves as an inhibitor of the same. Alloxanthine binds tightly to the active site of xanthine oxidase which prevents the oxidation of hypoxanthine to xanthine by the enzyme (34). This increases the concentration of hypoxanthine and decreases urate synthesis, as well as the rate of purine biosynthesis (Fig. 7B).

1.6.6. Purine salvage in *Leishmania*

Unlike humans, *Leishmania*, and other members of the Trypanosomatidae family, are incapable of *de novo* synthesis of the purine ring from formate, glycine, or serine precursors, and therefore require an exogenous purine source for growth (35). The inability of *Leishmania* to synthesize the purine ring *de novo* was proven experimentally by Marr, Berens and Nelson in 1978 when they demonstrated a lack of incorporation of [^{14}C]-formate [^{14}C]-glycine or [^{14}C]-serine by *L. donovani* into ATP or GTP pools (35). Other experiments detecting incorporation of [U-^{14}C] glucose into purine and pyrimidine nucleotide pools of *L. donovani* suggest that ample PRPP can be generated from glucose to sustain synthesis of purine nucleotides by the salvage pathway (35). Therefore the level of PRPP synthesis is not limiting to the growth of these parasites.

Leishmania and the trypanosomes rely completely on their purine salvage pathways to obtain purines from their mammalian hosts and have developed an extensive network of purine salvage enzymes that enable them to scavenge purines from their host environment. Nucleosides and nucleobases are taken up

through a variety of transporters on the parasite plasma membrane, where they are then funelled through the purine salvage pathway (Fig. 8). A plethora of protein activities involved within the purine salvage pathway of *Leishmania spp.* have been determined through radiolabel experiments and analysis using high pressure liquid chromatography, thin layer chromatography and spectrophotometry. The major purine salvage activities found in *Leishmania donovani* promastigotes include, but are not limited to: AK, IMPDH, ADSS, ASL, GMPS, APRT, HGPRT, guanine deaminase (GDA), and three purine nucleoside hydrolases. At least one of the hydrolases, inosine-uridine nucleoside hydrolase, (IUNH) is orthologous to mammalian IUNH, and it has the ability to cleave uridine, cytidine, xanthosine, inosine, adenosine and guanosine (36, 37). Of the other two hydrolases, one is specific for purine 2'-deoxyribonucleosides, and the other for inosine and guanosine, but not adenosine or xanthosine (36, 38, 39). Adenosine may also be cleaved by adenosine nucleosidease (40). *Leishmania* also express xanthine phosphoribosyltransferase (XPRT) and adenine aminohydrolase (AAH). Neither XPRT nor AAH are present in mammalian cells; therefore they are potentially good targets for selective chemotherapeutic agents. The differences between the purine salvage pathways of *Leishmania donovani* promastigotes and humans are highlighted in (Fig. 9).

The *Leishmania* purine salvage pathway is both complex and redundant because nucleobases and nucleosides that are transported into the parasites can be metabolized to multiple products before proceeding through the PRTs or AK

(Fig. 8). *Leishmania donovani* nucleoside transporter 1 (LdNT1) transports adenosine. Adenosine can either be directly phosphorylated by AK to form AMP or catabolized to adenine. The adenine produced may then be phosphoribosylated by APRT or deaminated to hypoxanthine and subsequently phosphoribosylated by HGPRT. Past studies have indicated that the majority of the adenosine taken up by *L. donovani* is converted to hypoxanthine before proceeding through the rest of the purine salvage pathway (35). *Leishmania donovani* nucleoside transporter 2 (LdNT2) transports Inosine and guanosine. After entering the cell inosine can be hydrolyzed by IUNH, or other nucleoside hydrolase, to form hypoxanthine, which is then metabolized by HGPRT. Some experimental results suggest that inosine may also be directly phosphorylated by a non-specific phosphotransferase, an enzyme which uses a substrate other than ATP as the phosphate donor, but the absolute existence and activity of such an enzyme remains to be proven (41). Guanosine is cleaved to guanine, the majority of which is then deaminated to xanthine by GDA. Xanthine is then phosphoribosylated by XPRT. The guanine that is not deaminated is phosphoribosylated by HGPRT. *Leishmania donovani* nucleobase transporter 3 (LdNT3) transports the purine bases adenine, hypoxanthine, guanine, and possibly xanthine. The majority of the adenine and guanine taken in by *L. donovani* are cleaved to hypoxanthine and xanthine, respectively, and utilized by HGPRT and XPRT (35).

1.6.7. Purine Salvage in Amastigotes vs. Promastigotes

Even though purine salvage within *Leishmania* promastigotes has been studied extensively, considerably less information is available about the purine salvage pathway in amastigotes, the stage of *Leishmania* that proliferates within mammalian macrophages, and ultimately causes leishmaniasis. The phosphoribosyltransferase activities in amastigotes are similar to those detected in promastigotes, with a relative phosphorylation of guanine>adenine>hypoxanthine>xanthine (38). Adenosine kinase (AK), possibly inosine kinase (or a phosphotransferase) and guanine deaminase activities have all been detected in both promastigotes and amastigotes (38).

Differences in *L. donovani* amastigote and promastigote adenine and adenosine metabolism have been suggested to occur. The results of past experiments led to the hypothesis that promastigotes metabolize adenine and adenosine differently than amastigotes. Adenine aminohydrolase (AAH, or adenine deaminase, adenase) activity but no adenosine deaminase (ADA) was detected in *L. donovani* promastigotes. Conversely, ADA, but no AAH activity was measured in amastigotes, and the level of AAH activity was used as an indicator to distinguish between promastigotes and amastigotes (35). Similarly, an activity that cleaves adenosine to adenine, possibly an adenosine phosphorylase, was detected in promastigotes but not in amastigotes (38). It may be inferred from these experiments that the two life stages of *L. donovani* metabolize adenosine and adenine differently, but these results are debatable (See Chapter 3 and Chapter 3 Appendix). In both promastigotes and

amastigotes hypoxanthine is ultimately formed from a portion of the adenine and adenosine that is metabolized by the parasites. This nucleobase is subsequently converted to IMP by HGPRT and funneled through the rest of the purine salvage pathway. The activities various purine salvage enzymes previously measured in promastigotes and amastigotes are summarized in Table 3. The differences that have been proposed to occur in the purine salvage pathways of *L. donovani* amastigotes and promastigotes are highlighted in (Fig. 10) (38).

Remarkably, early experiments to establish the purine salvage pathway in *L. donovani* promastigotes and amastigotes were all done using TLC, HPLC, spectrophotometric and scintillation counting experiments. Their findings have since been confirmed with far more sophisticated genetic and biochemical tools. Continuous evolution and advancement of science makes it possible to carry out a more thorough dissection of this pathway by employing genetic and molecular biology techniques. A genetic analysis of the purine salvage pathway in *Leishmania donovani* in which the proposed differences between promastigotes and amastigotes can be assessed is a major focus of this thesis.

1.6.8. Metabolism of purine analogs and isomers in *Leishmania donovani*

The mediocre selectivity of many members of their purine salvage pathway enables *Leishmania donovani* parasites to bind and metabolize several purine analogs and isomers, many of which deleteriously affect the parasites

(Fig. 11). *Leishmania* can convert most of the purine analogs and isomers into the equivalent nucleosides, which can then be incorporated into mRNA. The presence of non-native nucleosides within the mRNA causes it to break down, resulting in a net efflux of nucleosides into the cytoplasmic pool and an inhibition of protein synthesis (42).

Several compounds are phosphoribosylated by either HGPRT or APRT to become isomers or analogs of IMP. The subsequent metabolites of the IMP analogs and isomers can inhibit ADSS, which blocks the formation of AMP. They can also inhibit GMP reductase, which prevents the conversion of GMP to AMP, or may bind IMPDH and block the conversion of AMP to GMP (42). Ultimately the purine analogs and isomers may be converted to nucleotide analogs that can be phosphorylated and incorporated into mRNA. 4-Hydroxypyrazolo[3,4-d]pyrimidine (Allopurinol, or HPP), a hypoxanthine isomer, and 6-mercaptopurine, a hypoxanthine analog, are both phosphoribosylated by leishmanial HGPRT to become either an IMP isomer or analog, respectively (42, 43). Similarly, 4-aminopyrazolo[3,4-d]pyrimidine (4-APP), an adenine isomer, is phosphoribosylated by APRT to form an IMP isomer. All three of these compounds are eventually incorporated into mRNA as 4-APP triphosphates (43, 44).

Several adenosine analogs, including 9-deaza-adenosine, 7-deaza-adenosine (tubercidin) and Formycin A are phosphorylated by adenosine kinase, and ultimately incorporated into mRNA as nucleoside analogs. 3-

deazaguanosine, a guanosine isomer, and allopurinol ribonucleoside, an inosine isomer, as well as the inosine analog thiopurinol ribonucleoside are phosphorylated by a non-specific nucleoside phosphotransferase and incorporated into mRNA (42). Another inosine analog, Formycin B, is initially phosphorylated by a nucleoside phosphotransferase, then metabolized to Formycin A 5-mono-, di- or triphosphates and incorporated into mRNA as the cytotoxic adenosine analog Formycin A 5'-triphosphate (41). Finally, 9-deaza-inosine, another inosine analog, is metabolized to both ATP and GTP analogs after its initial conversion into an IMP analog (45). The presence of both ATP and GTP analogs suggests that the IMP analog formed by the phosphorylation of 9-deaza-inosine may be a substrate for ADSS as well as IMPDH (45).

9-Deaza-inosine initially showed great promise for use as an antileishmanial compound, until it was discovered that the spider monkeys in which it was tested developed fatty livers after 27 days of treatment (46). Consequently, this finding quenched the desire to use 9-deaza-inosine in humans as a treatment for leishmaniasis. Many of the aforementioned purine analogs and isomers have been tested for use as antileishmanial compounds, and several proved to be leishmanicidal, or growth inhibitory, at low doses. They are also useful *in vitro* selective agents for detecting missing, dysfunctional or mutant protein products within *Leishmania* parasites (40). However, because most of these compounds are metabolized to the same end products in humans as they are in *Leishmania* (46), it is too dangerous to use them as

chemotherapeutic agents, with the exception of allopurinol and allopurinol riboside (45).

1.6.9. Allopurinol

The anti-leishmanial activity of allopurinol was discovered over 30 years ago. Its oral bioavailability and use as a treatment for gout raised hopes that allopurinol may be effective against visceral leishmaniasis, but the results were disappointing. Allopurinol, which acts as a pro-drug by being selectively incorporated into nucleotide intermediates and nucleic acids in the parasite, was demonstrated to decrease the growth rate of *Leishmania donovani* promastigotes and prevent their transformation from promastigotes into amastigotes *in vitro* (47). In *Leishmania,* HGPRT initially converts allopurinol to the IMP analog allopurinol ribonucleoside 5'-monophosphate (26), a metabolite that competes with IMP for binding to ADSS and GMPR that is eventually incorporated into mRNA as 4-APP, which decreases protein synthesis. Allopurinol is leishmanistatic rather than leishmanicidal and decreases the growth rate of *L. donovani.* Growth inhibition by allopurinol can be reversed by the addition of adenine, hypoxanthine or inosine to the parasite growth medium (48). Furthermore, the affinity of *L. donovani* HGPRT for hypoxanthine (6.7 μM) is over 100 fold greater that for allopurinol (714 μM) (49), therefore the hypoxanthine that is salvaged from the host *in vivo* should effectively compete with allopurinol for

binding to HGPRT, thus decreasing the efficacy of the drug, especially as a treatment against visceral leishmaniasis.

Allopurinol has been used as a treatment for cutaneous leishmaniasis in the past, but its efficacy has decreased over time. Clinical trials of allopurinol in combination with other anti-leishmanial compounds, especially the antimonials, have shown more promising results for the treatment of cutaneous leishmaniasis (25). However, more recent interest in the allopurinol-antimonial combination has been for the treatment of canine leishmaniasis where significantly better results were found with drug combination therapy than with either drug alone (25).

Allopurinol-riboside, a metabolite of allopurinol, is more active *in vitro* against *Leishmania* than allopurinol. Like allopurinol, allopurinol-riboside is ultimately metabolized to the nucleotide analog of IMP, but by a different action: by direct phosphorylation from a nucleoside phosphotransferase (42). With allopurinol-riboside, growth inhibition reversal upon addition of adenine, hypoxanthine or inosine is not seen, a result that led to the study of this compound as an antileishmanial drug (48). In one study, treatment of patients with 5g/day of allopurinol-ribonucleoside for 28 days led to a 46% cure rate of cutaneous leishmaniasis. However, allopurinol-ribonucleoside is expensive and more difficult to synthesize than allopurinol, and is therefore not commonly used to treat cutaneous leishmaniasis (42).

1.7. Culture and Genetic Manipulation of *Leishmania*

1.7.1. *Leishmania* as a model system

Members of the Trypanosomatidae family, the largest family in the order Kinetoplastida, are responsible for a significant parasitic disease burden throughout the world. In particular, *Leishmania spp.*, *Trypanosoma cruzi* and *Trypanosoma brucei spp.* together infect approximately 30 million people world-wide, with an additional 530 million people at risk for infection, and cause a combined 144,000 deaths each year (3). *Leishmania* and the trypanosomes exhibit similar cell biology, therefore findings in *Leishmania* may be extensible other species within the Trypanosomatidae family. *Leishmania spp.* are a particularly attractive model organism to study because they are highly amenable to genetic, biochemical and molecular biological manipulations. *Leishmania* promastigotes can be propagated axenically and continuously under defined growth conditions, which facilitates pharmacological manipulation of the culture medium and enables isolation of parasites and their subcellular components in a state that is uncontaminated by mammalian cell debris (50). In addition, there are good *in vitro* and *in vivo* models relevant to host-parasite interaction. There are a wide variety of inbred and knockout mouse strains, some good models for cutaneous infections and others good for visceral infections, which are useful tools for investigating parasite virulence (19).

The availability of axenic (51, 52) and macrophage culture systems (53) for propagation of *Leishmania* amastigotes provides a convenient way to validate inferences, developed from *in vitro* studies on the promastigote, within the infective stage of the parasite. A few strains of *Leishmania* can be transformed axenically into amastigotes by adding and/or deleting components from the culture medium, lowering its pH and increasing the growing temperature. These conditions mimic those encountered by the parasite within the phagolysosome of the macrophage cells. Although these axenic amastigote-like forms (ALFs) may not be 100% identical to amastigotes isolated from lesions, most properties are reproduced, making the ALFs a powerful tool for studies of amastigote biology and gene expression (19). In addition, primary mouse macrophages can be made available for *in vitro* infectivity studies and other macrophage-like cell lines can also be cultured *in vitro* to facilitate analysis of parasite infectivity, virulence and survival in the appropriate cell type. Thus, *Leishmania* offer a relevant and experimentally manipulable model to study the basic biochemistry of a family of infectious agents that cause a variety of diseases.

1.7.2. Transfection technology

A vector for stable transfection of *Leishmania* was developed in the Beverley laboratory in the early 90's (50, 54). This vector is one of the most powerful tools available for analysis of the function of kinetoplastid genes within the organisms themselves. With this technology, it is possible to generate

knockouts, create and complement mutants, and over express both foreign and self genes in the parasites (55). Transfection technology is particularly useful because, in theory, inactivation of any locus essential for growth *in vivo*, but not *in vitro*, could be a suitable method for developing attenuated parasite strains that could be used to vaccinate against virulent parasites (56). Transfection technology can be utilized for targeted gene replacement, complementation experiments, inducible expression systems and RNA interference (55).

Transfection technology in *Leishmania* began with transient transfection, which is useful for rapid analysis of a variety of functions such as transcription initiation, post-transcriptional regulation and protein targeting. However during a transient transfection a maximum of 2% of the transfected cells successfully express the product of interest, therefore the resulting genotypes and phenotypes are quite difficult to analyze. The minimal vector for transient gene expression in *Leishmania* consists of a circular plasmid containing a signal for trans-splicing of the mRNA, followed a by a reporter gene. Transient transfection techniques quickly evolved, and the creation of permanently transformed cell lines was made possible by the inclusion of a selectable marker on the transient transfection vector (54).

1.7.3. Genetic Manipulation

The ability of *Leishmania* to undergo homologous gene replacement at high efficiency, in combination with transfection technology, has provided a

powerful vehicle for the genetic manipulation of *Leishmania* parasites, and affords a mechanism to test gene function via creation and characterization of metabolic null mutants (54, 57-59). There is no evidence that *Leishmania* undergo random exchange of genetic material with each other in the wild. Therefore, sexual crosses are not feasible for genetic manipulation of these organisms. However, there are many other tools available for genetic manipulation of *Leishmania* and these are well developed, including a variety of selectable markers, reporter genes and expression vectors (Table 4). These are described in detail below in order to provide a thorough understanding and rationale behind the methods I have chosen to use throughout the course of my thesis studies.

1.7.4. Targeted Gene Replacement

An invaluable technique available for genetic manipulation of *Leishmania* parasites is targeted gene replacement (TGR). TGR allows for complete elimination of a gene and its product, and therefore enables a researcher to evaluate the consequences of gene elimination. TGR is more efficient in *Leishmania* than inducible systems, especially for the purpose of completely eliminating the protein product of a gene, and was therefore the method of choice for completing the main goals of my thesis.

A targeted gene replacement (TGR) vector is a circular piece of DNA made up of three main components, vector DNA, specific host DNA and a

selectable marker. For use in *Leishmania*, a TGR construct usually, but not always, contains a backbone of leishmanial DNA flanked by two multiple cloning sites that surround a selectable marker, e.g. a drug resistance gene. The specific leishmanial DNA contained within the multiple cloning sites is either 100% homologous or highly equivalent to the 5' and 3' flank of the untranslated regions immediately preceding and succeeding the coding sequence of the *leishmanial* gene of interest, respectively. The flanks are amplified using PCR and pertinent restriction sites are added during this time. This allows ligation of the 5' flank into one multiple cloning site of the TGR vector in the proper direction and orientation and the 3' flank into the other. The TGR cassette is excised from the TGR vector using one enzyme that cuts at the upstream end of the 5' flank and another that cuts at the downstream end of the 3' flank. This excised cassette consists of a selectable marker surrounded by the 5' and 3' flanks of the leishmanial gene of interest, with an additional portion of leishmanial sequence between the flanks and marker. TGR within *Leishmania* is then possible through use of the linear TGR cassette. However, because *Leishmania* parasites are diploid at most loci, they may need to be subjected to two rounds of successful TGR in order to completely replace a gene (56, 57).

During the first round of targeted gene replacement, the TGR cassette is transfected into the parasites via electroporation. This high-voltage electrocution enables the TGR cassette DNA to enter the nucleus. Because the TGR cassette is linear, the flanks of the gene of interest align in the proper orientation with

homologous regions of the host chromosomal DNA. After this alignment, crossover between host DNA and TGR cassette occurs, and homologous recombination between the two entities is completed during cell division. The gene of interest is then replaced by the selectable marker, and the flanking regions remain as they were because they were replaced by homologous sequences during recombination (Fig. 12). The DNA that does not undergo homologous recombination is degraded by the parasite, and the cells in which TGR has successfully occurred can be selected for by adding the substrate of the selectable marker, which may be a drug at a concentration that would otherwise kill wild type, non-transfected parasites. A second round of TGR can subsequently be carried out; this round would use an additional selectable marker within an equivalent TGR cassette to replace the other copy of the *leishmanial* gene of interest, therefore abolishing production of the gene's protein product when both rounds of TGR have been successful. A variety of positive selectable markers are available for stable transfection using TGR, as well as several episomes for transient transfection with positive and negative selection (Table 4) (60).

If, after two rounds of transfection, a homozygous gene knockout is deleterious or lethal, clones with a knockout genotype might not be obtained. The study of essential genes offers a significant challenge to the genetic manipulation of *Leishmania* since the parasites have demonstrated their ability to circumvent this manipulation. It is difficult to know whether a gene is essential or

if one has encountered experimental error or technical difficulties. In some experiments, attempts to use TGR to knockout essential genes have yielded the planned replacements but are accompanied by expansion of the target gene number through changes in chromosomal number (19). Studies in *Leishmania* have also revealed several other events that can occur. The first marker may be replaced with the second, the DNA may integrate upstream or downstream of the gene of interest, or the marker might be preserved as an episome (55).

1.7.5. Episomal Complementation

If a gene is thought to be essential, a circular episomal DNA component may be transfected in to compensate for the production of the protein product while attempts are made to knock out the second wild type allele (55). In theory, the episome can be cured (eliminated from the parasites) by removing the drug for which it encodes resistance, or by using a negative agent that selects against parasites that retain the episome. If curing occurs, then the gene is not essential. However, if curing does not occur, then the gene may or may not be essential. An episome may also be used to add back a gene after it has been eliminated. This is particularly useful in studying protein function and localization (61, 62).

1.7.6. LOH

Leishmania possess the machinery to undergo loss of heterozygosity (LOH). LOH occurs when one copy of a gene has been knocked out and the

parasites randomly lose the second copy of the gene. It is not known whether the parasites lose only the gene, part of the chromosome, or an entire chromosome during this phenomenon. However, LOH may arise as a second homologous recombination within the first round of TGR, resulting in cells bearing two copies of a selectable marker, or by an accidental chromosomal segregation (55).

Cytotoxic purine analogs, such as HPP, APP, tubercidin and Formycin B, can be used as negative selective agents to detect the LOH phenomenon of genes such as *HGPRT*, *APRT*, *LdNT1* and *LdNT2*, respectively. Adding these drugs to parasite media does not cause LOH, but parasites that have undergone LOH are able to survive in the presence of the drug, and those that haven't die. In the Ullman laboratory APP and HPP have been used to select for LOH of *APRT* and *HGPRT*. Tubercidin and Formycin B, which are toxic adenosine and inosine analogs, respectively, have been used to select for the LOH of the transporter genes *LdNT1* and *LdNT2*.

1.7.7. Mutagenesis and complementation

Parasites can be subjected to mutagenic compounds, such as *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG), and selected for decreased susceptibility to a toxic compound or the inability to metabolize a substrate. Experiments of this nature can be used to investigate substrate transport and metabolism (40, 63). Parasites can also be mutagenized and selected for the loss of a particular

function. The genes responsible for the defects can be cloned after transfecting the mutant with a *Leishmania* genomic cosmid DNA library and selecting for recovery of the function that was lost (55, 64).

1.7.8. Inducible expression system

Transfection of an inducible system allows a tetracycline responsive (or other inducible) promoter to be used for regulation of gene expression, which enables control of the over- or under- expression of parasite genes (55). A tetracycline repressor/operator system has been adapted for use in *Leishmania*, but absolute regulation of the inducible machinery remains to be perfected (65, 66). Even though the repressed gene expression is substantially lowered, there is still a low level of read-through of the transcript and subsequent expression of the protein encoded by the gene of interest (55). Because this system cannot be used to study the effects of a gene deletion in *Leishmania*, it is most useful in studying genes with essential functions whose deletion would be otherwise lethal (55).

1.7.9. RNA interference

RNA interference (RNAi) occurs when the expression of double-stranded RNA decreases the abundance of cognate mRNAs. Large double stranded RNAs are cleaved into smaller 21-24 mers (termed small interfering (si) RNAs) which then target cognate mRNA messages for degradation (67). RNAi has

shown good promise as a strategy for reducing RNA expression for subsequent genetic analysis of *T. brucei* and *C. elegans*, but has largely failed to yield substantial results in *Leishmania* and *T. cruzi* (67-69). On the whole, attempts to transfect double-stranded RNA or si RNAs, either stably or transiently, into *Leishmania* parasites have not reduced mRNA expression for the target gene; nor have these attempts significantly changed observable phenotypes (70). It is possible that RNAi fails to work in *Leishmania* because these parasites appear to express significant levels of endogenous double stranded RNAs, including those encoding DHFR-TS and histone 1 (55) without any deleterious effects. *Leishmania* lack the mobile genetic elements that are predicted to be involved in RNAi and possess only a single Argonaute gene, LMPWI1. Argonaute proteins have been demonstrated to be involved in RNA silencing in *C. elegans*, *Neurospora crassa*, *A. thaliana* and *D. melanogaster*. LMPWI1 is much closer in homology to the *T. brucei* Argonaute protein TbPWI1 than to TbAGO1. TbPWI1 is not believed to be involved in RNAi, whereas TvAGO1 is required for mitosis and chromosome segregation in *T. brucei* during RNAi (71).

1.8. Genetics and biochemistry of purine salvage in *L. donovani*

1.8.1. Purine Salvage Pathway Gene Cloning

One of the most intricate metabolic pathways in *Leishmania* parasites is their purine salvage pathway (Fig. 8). Aspects of the purine salvage pathway have been demonstrated to be different for *Leishmania* from what is known to occur in humans, and these are highlighted in Figure 9. Confirming the differences between the leishmanial and mammalian purine salvage pathways is one of the most important steps in rational chemotherapeutic drug design for targeting of leishmanial purine salvage proteins, and in developing effective drugs for use against *Leishmania* parasites. A thorough genetic dissection of the purine salvage pathway of *Leishmania donovani* has thus been a major goal of my thesis. Information gained through a genetic dissection of the *Leishmania donovani* purine salvage pathway may provide a portion of the requisite knowledge necessary to design more efficient chemotherapies or vaccines to treat and prevent leishmaniasis than have been available to date.

Several genes encoding proteins within the *L. donovani* purine salvage pathway have been cloned and sequenced using a plethora of cloning strategies. *IMPDH*, *APRT*, *HGPRT*, *XPRT* and *AK* were all cloned prior to the availability of the fully sequenced and partially annotated *Leishmania major* genome on-line database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/l_major/omni), the existence of which currently makes it relatively simple to clone genes from *Leishmania spp*. The cloning of each of these purine salvage genes is described briefly below because my thesis project would not have been possible without the

work that was done by my predecessors, including gene cloning, sequencing and the construction of numerous TGR constructs.

1.8.2. *IMPDH*

In order to isolate the *IMPDH* gene an *L. donovani* genomic library consisting of 16-24 kb fragments of DNA contained within the bacteriophage vector EMBL3 was screened with a mouse *IMPDH* cDNA probe. Positive plaques were purified, the isolated bacteriophage DNA was digested, and a 2.3 kb EcoRV/PstI fragment that hybridized to the mouse *IMPDH* cDNA was identified by Southern analysis. The fragment was purified, subcloned into the pBluescript vector and transformed into XL1-blue competent bacteria. DNA containing the *IMPDH* gene was purified and sequenced. *Leishmania donovani IMPDH* was found to be a single copy gene encoding a 514 amino acid protein, that is 52.5% identical to human IMPDH (40).

1.8.3. *APRT*

L. donovani APRT was isolated from a cosmid library by cross hybridization to a 242 base pair fragment of *APRT* that had been amplified from reversed transcribed RNA using PCR. A 5.4 kb BamHI/XbaI fragment encompassing the entire coding portion of *APRT* was ligated into pBluescript, transformed into competent bacteria and purified DNA was sequenced. The *APRT* genomic sequence revealed an open reading frame of 711 nucleotides

that encodes a 237 amino acid polypeptide. Recombinant APRT protein expressed in and purified from *E. coli*, has a K_m for adenine of 3.9 +/- 2.3μM and no affinity for hypoxanthine, guanine or xanthine (72).

1.8.4. *HGPRT*

The isolation of *L. donovani HGPRT* was initiated by N-terminal sequencing of purified HGPRT protein. A non-degenerate sense primer encoding the *L. donovani* mini exon and a degenerate antisense oligonucleotides that had been constructed to the last 10 amino acids of the HGPRT N-terminal sequence were employed to PCR-amplify a 163 base pair *HGPRT* DNA fragment from reverse transcribed RNA. The PCR product was then used as a probe to screen a cosmid library, and a positive cosmid was isolated and analyzed by Southern blotting. A 3.5 kb EcoRI fragment containing the *HGPRT* gene was subcloned for sequence analysis which revealed an open reading frame of 633 nucleotides encoding *HGPRT*. The *HGPRT* gene cloned is a single-copy gene that produces an HGPRT protein product of 211 amino acids and exhibits homology with other HGPRTs (49). Recombinant HGPRT protein purified from *E. coli* recognizes hypoxanthine, guanine and allopurinol, but not adenine or xanthine as a substrate (49). Complementation of purine *SΦ609 E. coli*, a strain that is auxotrophic for purines and lacks the bacterial PRT enzymes, HPRT and XGPRT, with *L. donovani HGPRT* restores growth of the bacteria on guanine or hypoxanthine. Complemented cells can not grow on 6-thioguanine or 6-

mercaptopurine, 2 cytotoxic substrates of HGPRT (35, 49). *L. donovani* HGPRT also phosphoribosylates HPP. Apparent K_m values of 6.4 μM, 9.9 μM, and 714 μM were calculated for hypoxanthine, guanine and HPP, respectively, and k_{cat} values for hypoxanthine and guanine were determined to be 5.7 s^{-1} and 12.1 s^{-1} (49).

1.8.5. *XPRT*

A directionally cloned *L. donovani* cDNA library was subjected to *in vivo* excision and electroporated into *SΦ609 E. coli*. The transformed *E. coli* were plated first on LB agar, then on LPI agarose plates that were supplemented with 150 μM xanthine. Plasmid DNA was obtained from the colonies that grew on xanthine and the ends of the inserts were sequenced. An *L. donovani* cosmid library was screened using the *XPRT* cDNA as a hybridization probe. A 4.3 kb EcoRI fragment encompassing both *HGPRT* and *XPRT* was subcloned from one purified cosmid into pBluescript and sequenced. *XPRT* was found to be a single-copy gene, encoding a 241 amino acid protein that is 33% identical to HGPRT. *XPRT*, which is thought to be the product of a gene duplication of *HGPRT*, is located approximately 2.2 kb downstream from *HGPRT* (59). XPRT kinetics were determined by over-expressing the *XPRT* gene in *Sφ609 E. coli*. The recombinant XPRT protein purified from these cells has a K_m value of 7.1 ± 2.3 μM for xanthine and a k_{cat} value of 3.5 ± 1.5 s^{-1}. Hypoxanthine and guanine are also recognized by the recombinant XPRT, although inefficiently, with K_m values

of 448 ± 97 and >100 μM, respectively. However, the k_{cat} value obtained for hypoxanthine is 2.6 ± 0.2 s^{-1}, a value comparable to that measured for xanthine. In contrast, the catalytic efficiency for guanine phosphoribosylation by XPRT is approximately 0.003 s^{-1}, ~3 orders of magnitude lower than the k_{cat} values for either xanthine or hypoxanthine (59). These data indicate that XPRT has approximately the same turnover rate for xanthine and hypoxanthine, but that it is much less efficient in metabolizing guanine.

1.8.7. *AK*

Purified AK protein was subjected to digestion with either CNBr or cleavage by endopeptidase LysC. The generated peptide fragments were separated by reverse phase HPLC and well-resolved peaks were analyzed using an automated sequencer. Four peptide sequences of approximately 16-26 amino acids in length were sequenced. A DNA probe was generated by PCR-amplifying a genomic DNA template using oligonucleotides designed against two short regions of the peptide sequences, and used to screen an *L.* donovani genomic library that was packaged in bacteriophage λGEM-11. DNA from one phage was purified and analyzed by Southern blotting. A 3.2 kb fragment that hybridized with the DNA probe was purified from agarose, ligated into pBluescript, transformed into competent cells and the plasmid DNA isolated from the transformed bacteria was sequenced. The 1035 base pair open reading frame of *AK* encodes a 345 amino acid protein. Expression of *AK* in *E. coli*, and

purification of recombinant AK protein, showed K_m values for adenosine and ATP to be 11μM and 31 μM , respectively (73).

1.8.8. Genomics

Efforts to clone and sequence several of the other purine salvage genes are currently underway in the Ullman laboratory. The process by which gene cloning occurs today is much easier than it used to be because of the completion of the Tri-trypanosome genome-sequencing project, wherein the genomes of *L. major*, *T. brucei* and *T. cruzi* were sequenced in full (74). Candidate genes in *Leishmania major* can be identified by searching for sequences that align with genes that have been annotated in one of the *Trypanosoma* species or in other organisms such as *H. sapiens, S. cerevisiae* or *E. coli*. The gene sequence from the *L. major* database can then be used to design PCR primers to generate a product with which to probe an *L. donovani* cosmid library, making isolation of the corresponding *L. donovani* gene possible.

The recent completion of the *Leishmania major* genome sequence (74) has helped advance the work of my thesis project. I have used the database to identify approximately twenty other genes within the purine salvage pathway, many of which have been cloned from *L. donovani*. The *Leishmania* genome network consortium sequenced the *Leishmania* portion of the Tri-trypanosome sequencing project. The network includes the Sanger Institute, U.K., Seattle Biomedical Research Institute, Seattle, and EULEISH (European *Leishmania*

major genome sequencing consortium). *L. major* Friedlin was the species of *Leishmania* chosen for the sequencing project. The *L. major* Friedlin genome has an estimated size of 33.6 megabases with a karyotype of 36 chromosomes. The database contains all *L. major* genes that have been assigned, either definite (as proven by experimental characterization or published) or putative identities, which are usually determined by their degree of homology with proteins of the same function from other organisms. Other items included within the database for each definite or putative protein include a map detailing on which chromosome the gene can be found, as well as sequences and genes that lie up to 10 Kb either side of the selected gene. Predicted peptide properties (i.e. prediction of putative signal peptides and trans-membrane domains) are also included.

1.9. Contributions of this thesis

1.9.1. Genetic analysis of the purine salvage pathway of *Leishmania donovani*

Previous biochemical, genetic and radiolabel experiments implicated APRT, HGPRT and AK as key players within the *L. donovani* purine salvage pathway and favored HGPRT as the enzyme through which most of the purine flux proceeds. To initiate a genetic dissection of the purine salvage pathway, the

Ullman laboratory created mutant strains of *L. donovani* deficient in these three purine salvage enzymes in every possible combination: three single mutants, three double mutants and one triple mutant (Fig. 13). The strains generated include *ak⁻*, *aprt⁻*, *Δhgprt*, *Δxprt*, *Δaprt/ak⁻*, *Δhgprt/ak⁻*, *Δhgprt/Δaprt* and *Δhgprt/Δaprt/ak⁻*, (58, 75). The *ak⁻* cell line was selected after wild type cells were chemically mutagenized and selected for growth on 20 μM tubercidin, a cytotoxic adenosine analog that is metabolized by AK (40), and the null mutants were generated within a wild type or the *ak⁻* background, using two rounds of TGR, or by single TGR with one drug resistance marker followed by selection for LOH (58, 75). These mutants retained the ability to proliferate in completely defined medium in which the sole purine source was any of the four naturally occurring purine nucleobases, hypoxanthine, guanine, xanthine or adenine, or the corresponding nucleosides (58). These data indicate that *L. donovani* that lack functional HGPRT, APRT and AK divert purines through a different pathway, and it was therefore hypothesized that the entire flux of the purine salvage pathway can proceed through XPRT. The *XPRT* gene from *L. donovani* was cloned, sequenced, and *Δxprt* knockouts were constructed in a wild type background. These mutants are able to salvage all purines except xanthine (59), thus indicating that XPRT is the only purine salvage enzyme that metabolizes this nucleobase. Further, the ability of all other purines to support growth of *ak⁻ /Δhgprt/Δaprt* cells can be explained by the fact that adenosine, adenine and inosine are converted to hypoxanthine by adenosine phosphorylase(or a

nucleoside hydrolase), AAH and IUNH, respectively, and that the hypoxanthine produced is then metabolized by XPRT. Finally, in the *Leishmania* purine salvage pathway, guanosine is catabolized to guanine by a nucleoside hydrolase, and guanine is converted by GDA to xanthine, which is then utilized by XPRT.

During the course of my thesis work I was able to generate an *ak⁻/Δaprt/Δxprt* mutant which can grow in any purine source except xanthine or xanthosine, indicating that the purine salvage pathway can also proceed entirely through HGPRT. Taken together, these results indicate that all purine nucleobases and nucleosides can be funneled to hypoxanthine, xanthine, or both, and implicate HGPRT and XPRT as the key players in the *Leishmania donovani* purine salvage pathway. Therefore, the entire flux of the pathway can proceed through HGPRT or XPRT. From this data we developed the hypothesis that one copy of HGPRT or XPRT is necessary and sufficient for survival of *L. donovani* promastigotes. Testing this hypothesis was one of the major focuses of my thesis project.

1.9.2. Goals of my thesis work

The overall objective of this thesis was to determine the importance of the phosphoribosyltransferases (PRTs) in the purine metabolism, viability and virulence of *L. donovani*. A genetic dissection of the purine salvage pathway was performed in order to achieve my long term goal: To expand existing knowledge about the biochemistry of purine salvage in *Leishmania* and to use this

information to better understand the ability of these parasitic organisms to cause disease. The studies conducted in this thesis will contribute to our understanding of the necessity of important purine salvage enzymes (APRT, HGPRT and XPRT) to the livelihood of *L. donovani* and ultimately help develop selective, rational treatment or prevention of leishmaniasis. The specific objectives were as follows:

1.9.2.a. To localize xanthine phosphoribosyltransferase (XPRT) and determine if its location is important for its function.

XPRT was localized in wild type *L. donovani* parasites using immunofluorescence analysis (IFA) with XPRT-specific antibodies. The putative glycosomal targeting signal was deleted from XPRT and an episomal DNA construct containing the mutant *xprt* gene was transfected into *Δxprt* cells. The mutant xprt protein was localized using IFA. Phenotypic and biochemical experiments were carried out to determine whether the localization of XPRT (or xprt) was important for its function. We concluded that wild type *L. donovani* XPRT is located in the glycosome and that the sub-cellular location of XPRT or xprt is not important for function.

1.9.2.b. To examine the purine salvage pathway of *L. donovani* amastigotes and to determine whether APRT, HGPRT or XPRT is required for transformation, infectivity and virulence.

Δaprt, *Δhgprt* and *Δxprt* mutants were generated in a virulent strain of *L. donovani*, LdBob. These parasites were first cultured as axenic amastigotes in order to allow for an assessment of their purine requirements within a completely controlled culture medium that was free of host cell components. Their ability to invade macrophages, transform into and proliferate as amastigotes within macrophages was then evaluated. The *Δaprt*, *Δhgprt* and *Δxprt* mutants were capable of transforming into axenic amastigotes, and could proliferate indefinitely as long as the appropriate purines were supplied. The nutritional requirements of the amastigotes were the same as their promastigote counterparts. In addition, wild type, *Δaprt*, *Δhgprt*, and *Δxprt* parasites could all infect and proliferate within bone-marrow derived macrophages in vitro at a level about half that of wild type. Near wild type parasitemia could be restored when these parasites were complemented with an episome encoding the PRT gene that had been eliminated. In addition, no differences in the purine salvage activities present in *L. donovani* promastigotes and amastigotes were revealed during these studies, however, the existence of several previously hypothesized purine salvage activities was proven genetically.

1.9.2.c.1. To insert the *Δxprt* mutation into *L. donovani* purine salvage mutants.

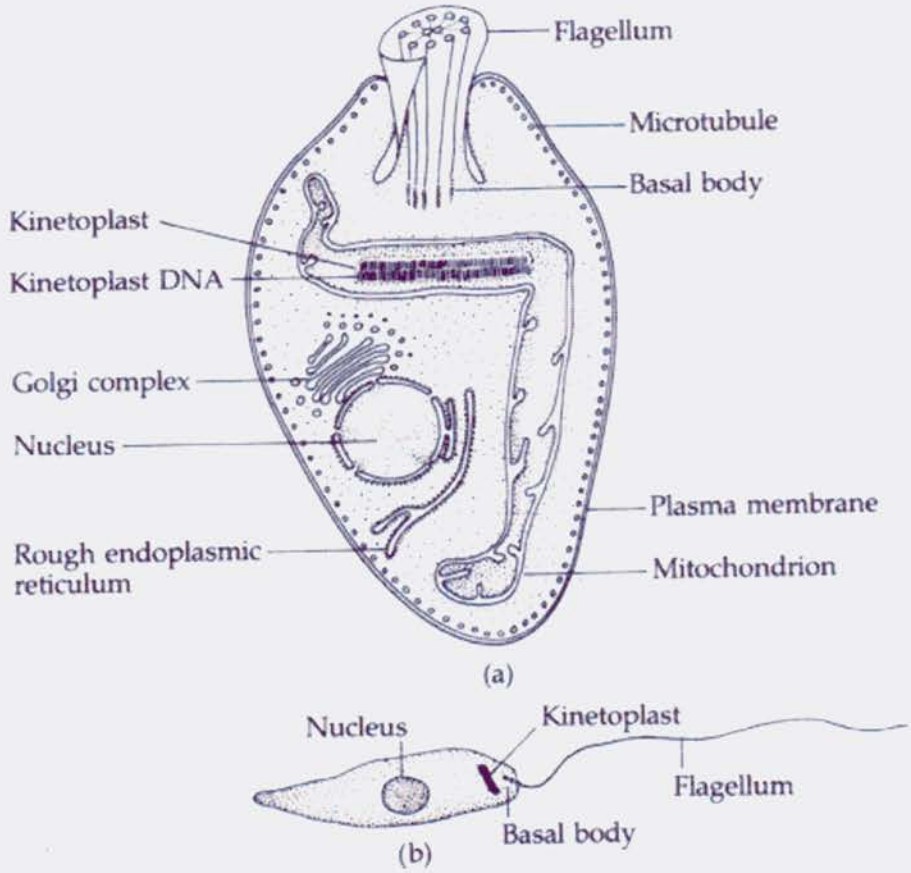
The goal of this specific aim was to genetically dissect the *L. donovani* purine salvage pathway and to further evaluate the importance of XPRT within

this pathway. This aim involved using targeted gene replacement techniques to insert the *Δxprt* mutation into several cell lines deficient in one or more purine salvage enzymes. Genotypic and phenotypic analyses were carried out on all cell lines generated under this specific aim. Many noteworthy results were observed while attempting to insert the *Δxprt* mutation into any cell line containing the *Δhgprt* mutation. For example, the insertion of one targeting construct caused rearrangement of the other TGR construct, rather than replacement of the other wild type copy of the gene of interest, drug resistance was achieved without a successful stable transfection, and a gene that was not the targeted gene of interest was eliminated during one round of TGR. Because of the numerous unusual results that were obtained while trying to insert the *Δxprt* mutation into any cell line containing the *Δhgprt* mutation, we formulated the hypothesis that one copy of HGPRT or XPRT is necessary and sufficient for the survival and proliferation of *L. donovani* promastigotes. However, this conclusion was based on negative evidence, and it was important to me that I definitively proved or disproved the hypothesis. I scrutinized the *L. donovani* purine salvage pathway to identify reactions within the pathway that could potentially be preventing me from generating the *Δhgprt/Δxprt* mutation within a wild type background, and. hypothesized that AAH was the primary suspect responsible for my failures. Finding a way to block its activity became the prerequisite for the final aim of my thesis project.

1.9.2.c.2. Generation of an *L. donovani Δhgprt/Δxprt* conditional mutant.

In order to block a potentially lethal metabolic event in a *Δhgprt/Δxprt* mutant, pharmacological intervention with deoxycoformycin (dCF), an inhibitor of *L. donovani* AAH (76), was necessary during the final round of transfection used to generate these parasites. The genotype of the *Δhgprt/Δxprt* mutants was determined by Southern blotting, confirmed by western blotting, and the growth and salvage phenotypes were characterized biochemically. The survival of *Δhgprt/Δxprt* mutants is dependent on the presence of dCF and either adenine or adenosine. They cannot grow on guanine, guanosine, hypoxanthine, xanthine or xanthosine, but surprisingly can grow, albeit meagerly, on inosine. These data establish genetically that either HGPRT or XPRT is essential for purine acquisition and parasite viability and that all exogenous purines are funneled to hypoxanthine and xanthine by *L. donovani* promastigotes. The *Δhgprt/Δxprt* mutant was also analyzed as an axenic amastigote and its ability to salvage , incorporate and metabolize the purines tested mirrored those of its promastigote counterpart. One of the most remarkable findings regarding the *Δhgprt/Δxprt* mutant was that they could not replicate within macrophages, thus establishing the possibility for the use of this strain as an anti-leishmanial vaccine.

1.10. Figures



Reproduced from (2)

Figure 1A. Drawing of a *Leishmania donovani* promastigote detailing the location of the kinetoplast.

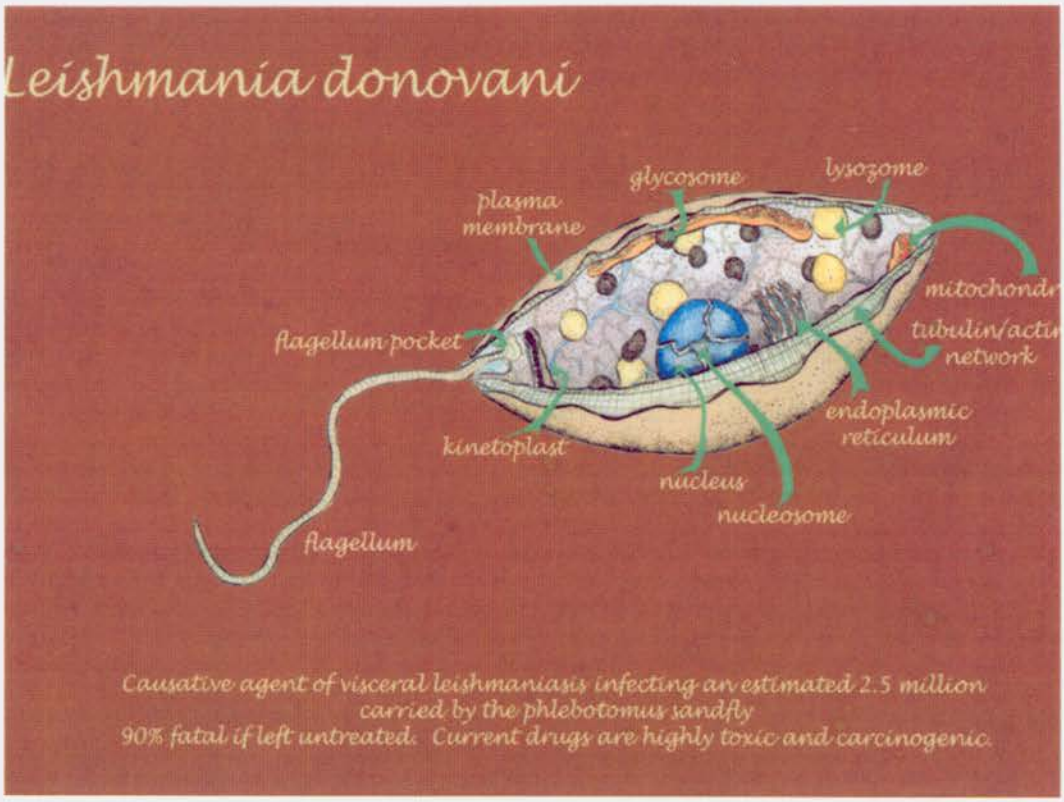


Figure 1B. Artist's rendition of the 3-dimentional view inside an *L. donovani* promastigote. The glycosome is labeled here. (By Nicolle Rager, 2001)

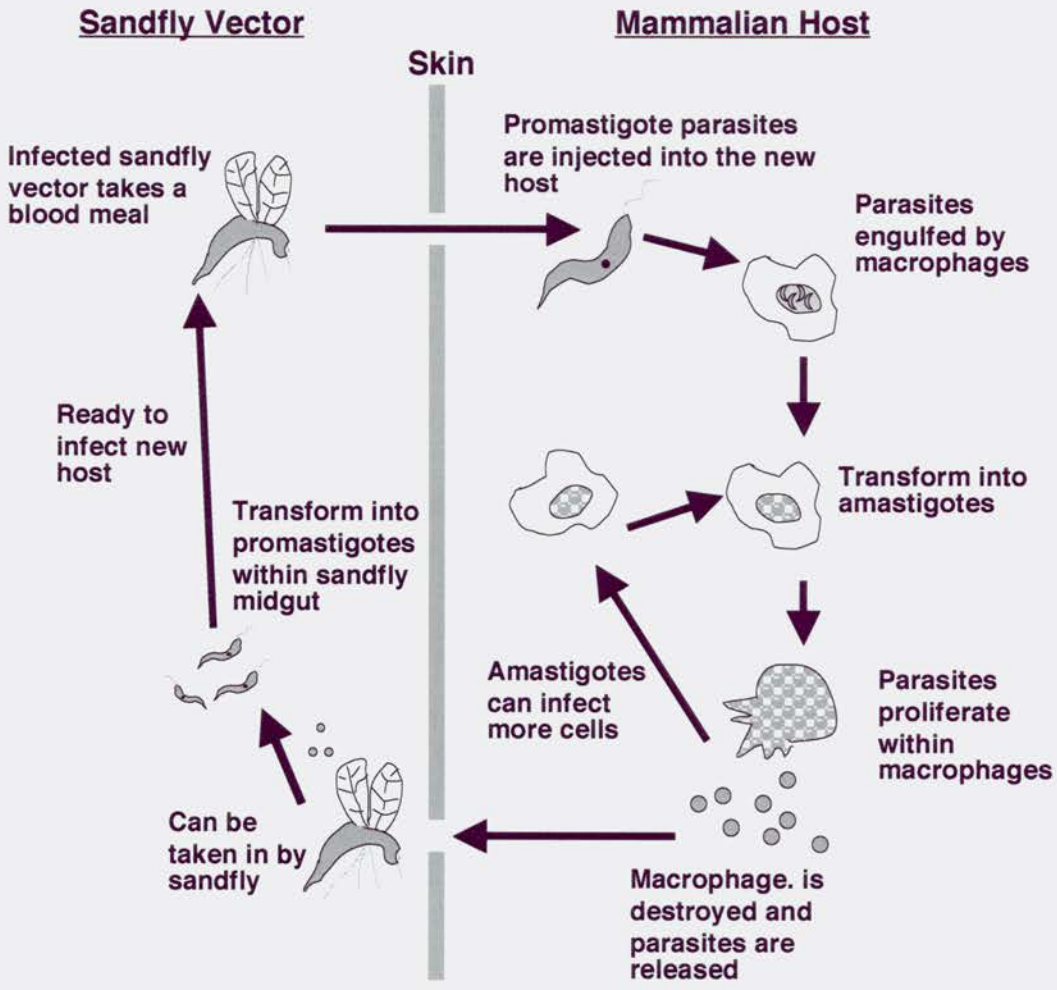


Figure 2. Life cycle of *Leishmania spp.* parasites. Host infection is initiated when an infected sandfly vector takes a blood meal.

Disease Manifestation	Old World*	New World **	Reservoir Host
Cutaneous	*L. major* *L. tropica* *L. aethiopica*	*L. mexicana* *L. amazonensis* *L. venezuelensis* *L. braziliensis* *L guyanensis* *L. panamensis* *L. peruviana* *L. pivonoi* *L. chagasi*	humans, rodents, cats dogs, cats, armadillos
Visceral	*L. donovani* *L. infantum*		humans, dogs, wild carnivores
Mucocutaneous	*L. archibaldi* (May actually be *L. donovani*)	*L. braziliensis*	Rodents, dogs cats, kinkajous

*Spread by *Phlebotomine* genus of sandfly
**Spread by *Lutzomyia* genus of sandfly
(3, 18, 23)

Table 1. Forms of leishmaniasis and the species of *Leishmania* that cause them. The species of *Leishmania* that are found in the Old world (Africa, India, Middle East and parts of Europe) versus the New world (South, Central and North America) and a few, select reservoir hosts are also listed.

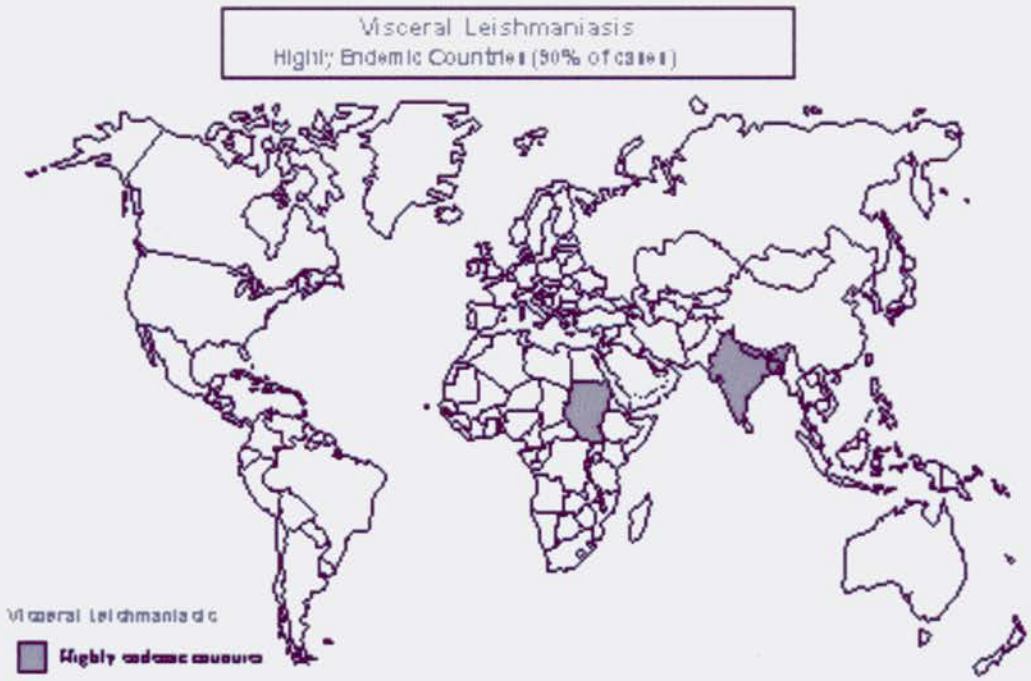


Figure 3A. Distribution of visceral leishmaniasis (3)

Figure 3B. Distribution of cutaneous leishmaniasis (3)

Figure 4. Historic representation of mucocutaneous leishmaniasis. Pre-Incan pottery has been found that depicts human disfigurement which bears a striking resemblance to the destruction of the mucosa membranes which can result from a mucocutaneous leishmaniasis infection (3).

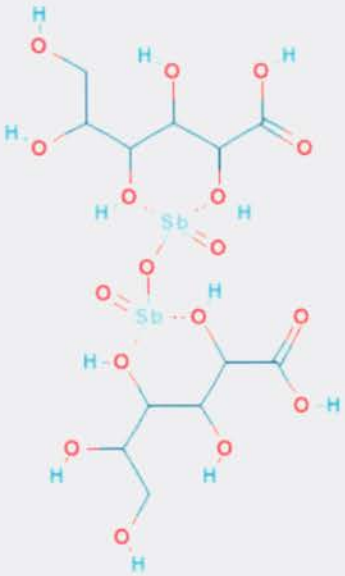
Generic name of drug (chemical type)	Mechanism of Action
Pentavalent antimonials: Meglumine antimoniate (Glucantime™), Sodium stibogluconate (Pentostam™)	Structure of sodium stibogluconate is still not known despite its use for over 50 years. Activated within the amastigote, but not in the promastigote, by conversion to a lethal trivalent form. Activation mechanism not known. Antileishmanial activity might be due to action on host macrophages.
Pentamidine (diamidine)	Accumlated by the parasite; effects include binding to the kinetoplast DNA. Primary mode of action uncertain.
Amphotericin B (polyene antibiotic)	Complexes with 24-substituted sterols, such as ergosterol in cell membrane, thus causing pores which alter ion balance and result in cell death.
Paromomycin (aminoglycoside antibiotic) (also known as aminosidine or monomycin)	In bacteria, paromomycin inhibits protein synthesis by binding to 30S subunit ribosomes, causing misreading and premature termination of mRNA translation. In *Leishmania*, paromomycin also affects mitochondrion.
Miltefosine (hexadecylphosphocholine)	Primary effect uncertain, possible inhibition of ether remodeling, phosphatidylcholine biosynthesis, signal transduction and calcium homeostasis.
Sitamaquine (8-aminoquinoline, originally WR6026) Imiquidmod (imidazoquinoline)	Unknown, might affect mitochondrial electron transport chain. Stimulates nitric oxide production from macrophages.

Table reproduced from (26)

Table 2. Mechanism of action of selected anti-leishmanial drugs

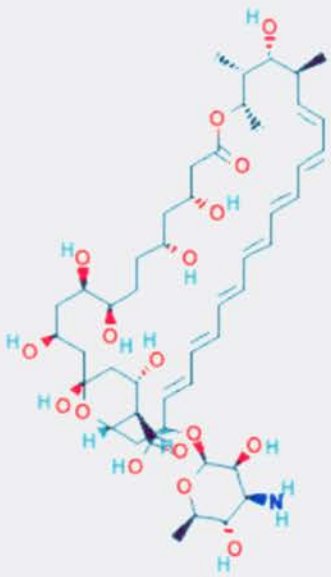
Figure 5. Structures of select anti-leishmanial drugs

A. Pentostam:

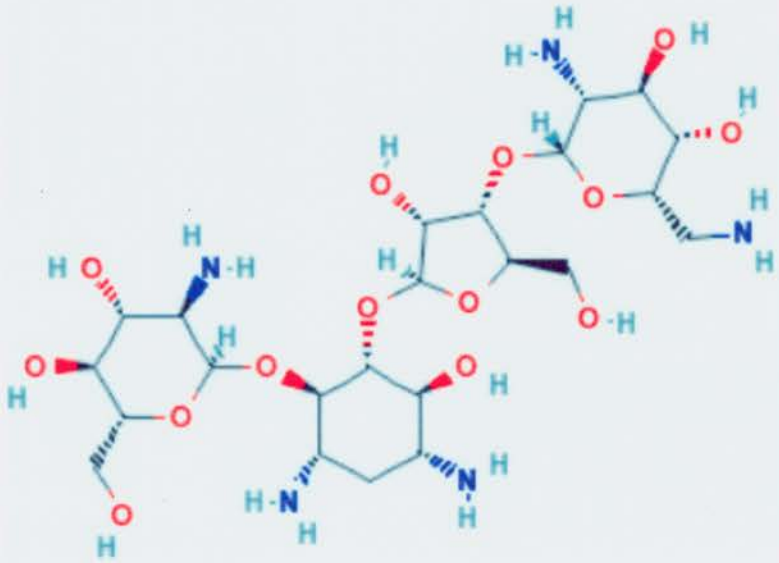


B. Meglumine antimoniate

C. Amphotericin B

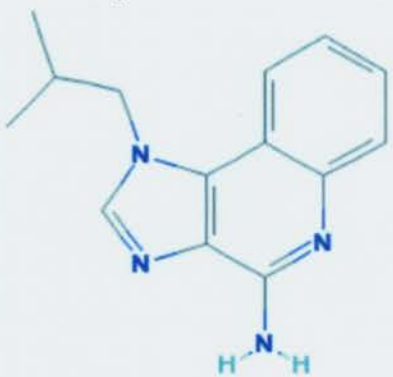


D. Paromomycin



E Miltefosine

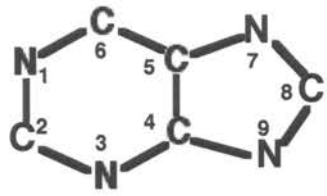
F. Imiquimod



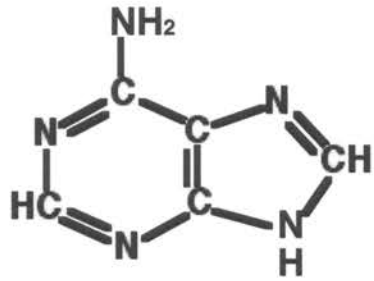
Structures from (77).

Figure 6. Structure of the purine ring, and an example of a nucleobase, a nucleoside and a nucleotide.

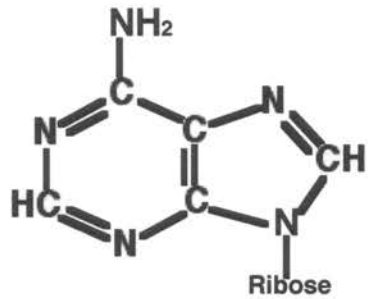
A. The purine ring. The positions of the carbon and nitrogen atoms that comprise the purine ring are numbered.



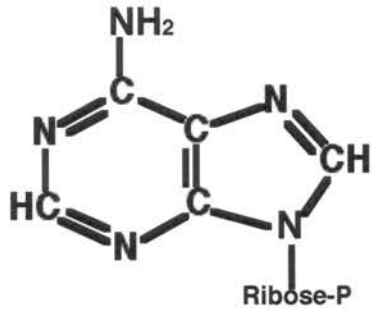
B. The purine base adenine

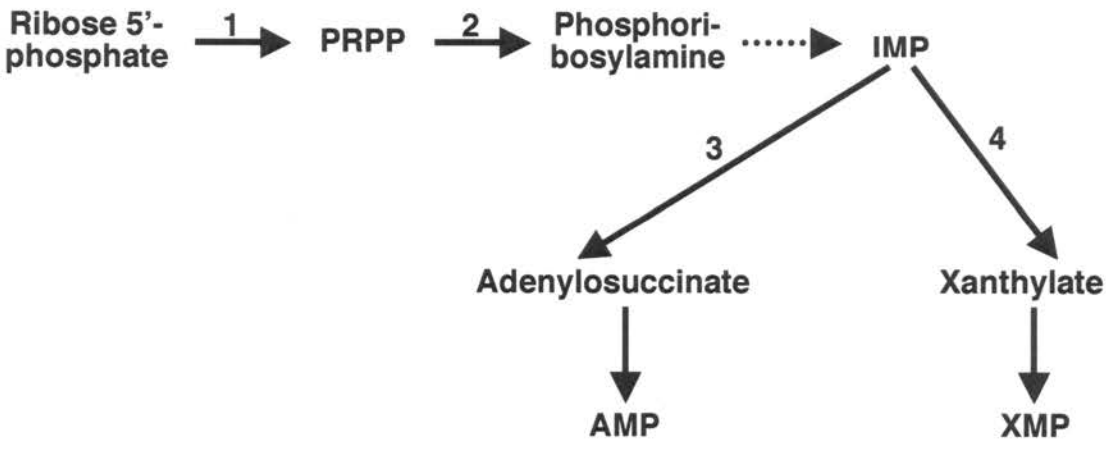


C. The purine nucleoside adenosine



D. The purine nucleotide adenosine monophosphate (AMP)



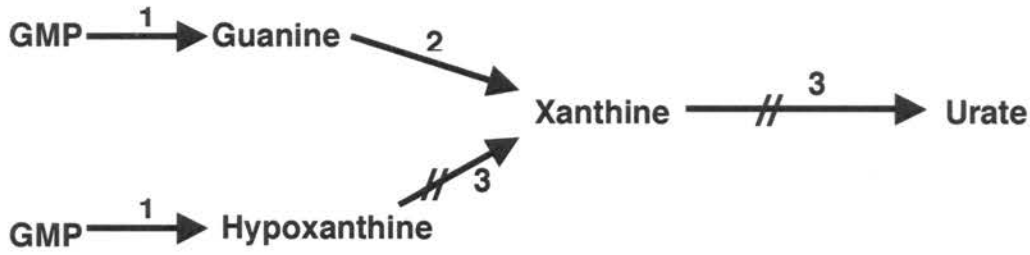


Enzyme identities and regulation

1. **PRPP synthetase (inhibited by IMP, AMP and GMP)**
2. **PRPP glutamine amidotransferase (inhibited by IMP, AMP and GMP)**
3. **ADSS (inhibited by AMP)**
4. **IMPDH (inhibited by GMP)**

Figure 7A. Regulation of purine nucleoside biosynthesis in humans.

Enzyme activities are numbered and the inhibitors of these activities are also listed.



Enzyme identities and inhibition

1. PNP
2. GDA
3. Xanthine oxidase (inhibited by allopurinol)

Figure 7B. Urate synthesis in humans is blocked by allopurinol. The steps inhibited by allopurinol are indicated with a (//) symbol.

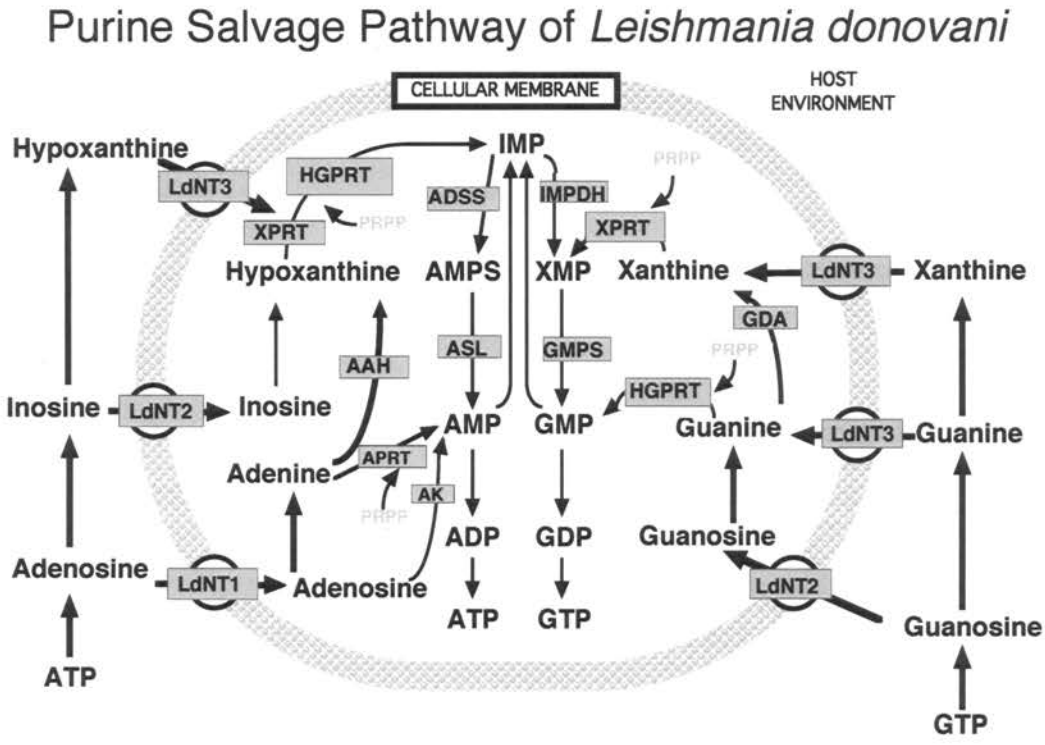
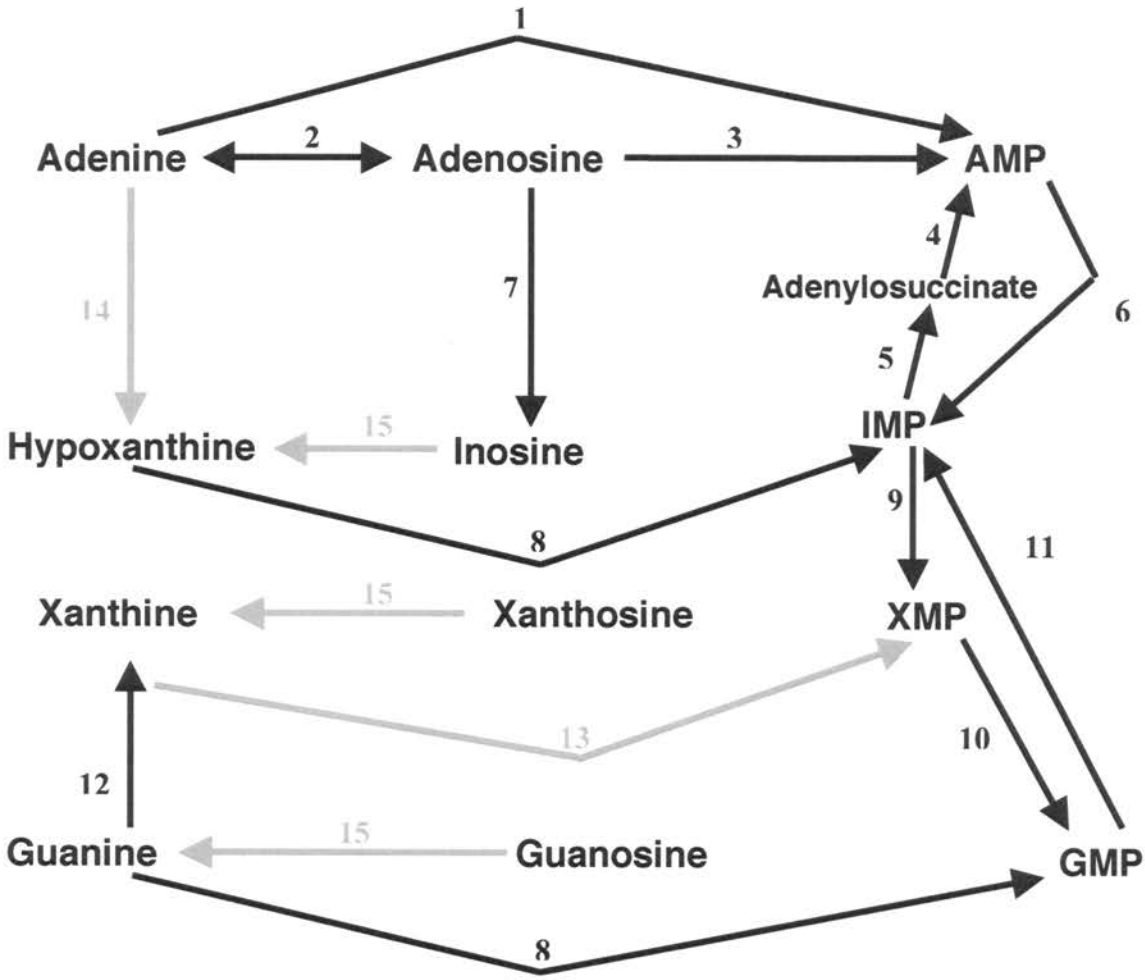


Figure 8. The proposed purine salvage pathway of *Leishmania donovani* promastigotes



Enzyme Identifications

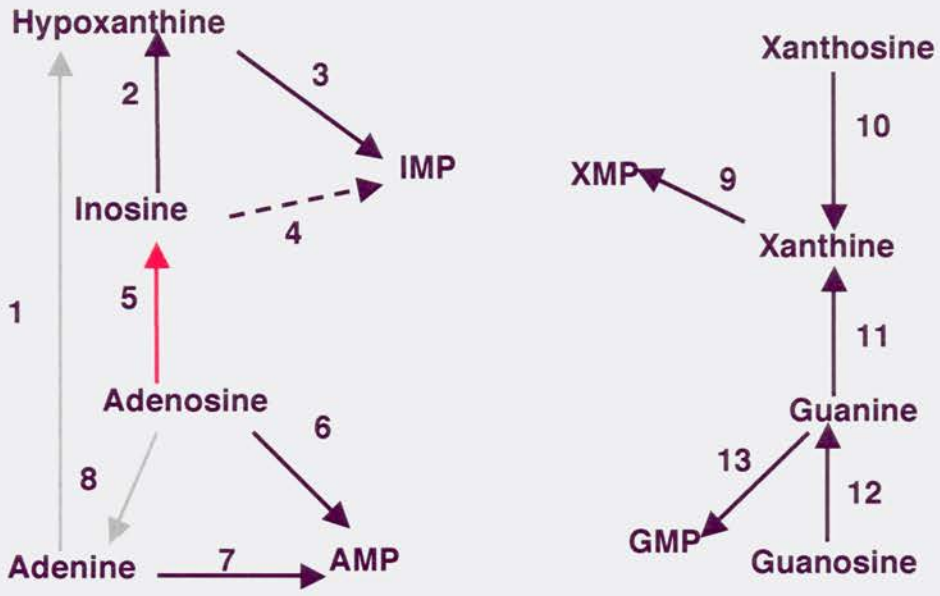
2. Nucleoside phosphorylase
3. Purine (adenosine) nucleoside kinase
4. Adenylosuccinate lyase
5. adenylosuccinate synthase
6. AMP deaminase
7. Adenosine deaminase
8. HGPRT
9. IMPDH
10. GMP synthetase
11. GMP reductase
12. Guanine deaminase
13. XPRT
14. Adenine deaminase
15. Purine nucleoside hydrolase **(or IUNH)**

Figure 9. Purine salvage pathways in *Leishmania spp.* vs. mammalian cells.

The differences found within the parasites are highlighted in gray. Reproduced from (39).

Enzyme	Activity (nmol min^{-1} mg^{-1} protein)	
	Promastigotes	Amastigotes
PRTs		
Guanine	150	47
Adenine	63	43
Hypoxanthine	41	31
Xanthine	18	1
Nucleosidases		
Inosine	140	80
Guanosine	75	54
Xanthosine	47	25
Adenosine	17	0
Nucleoside kinases		
Adenosine	.5	24
Deaminating		
Guanine	11	21
Adenine	25	0
Adenosine	0	68

Table 3. Purine salvage enzyme activities in promastigotes and amastigotes as identified in 1983. Reproduced from (38).



Enzyme Identities

1. AAH
2. IUNH
3. HGPRT
4. Inosine kinase (activity measured, but identity not confirmed)
5. ADA
6. AK
7. APRT
8. PNP
9. XPRT
11. GDA
12. NH
13. HGPRT

Figure 10. Purine Salvage Pathways of *L. donovani* promastigotes and amastigotes. Gray arrows indicate activities predicted to be exclusive to promastigotes, and a red arrow indicates an activity that is predicted to occur exclusively in amastigotes. The existence of an adenosine deaminase still remains to be genetically proven. Common activities are depicted with black arrows. (Adapted from (Looker, 1983)

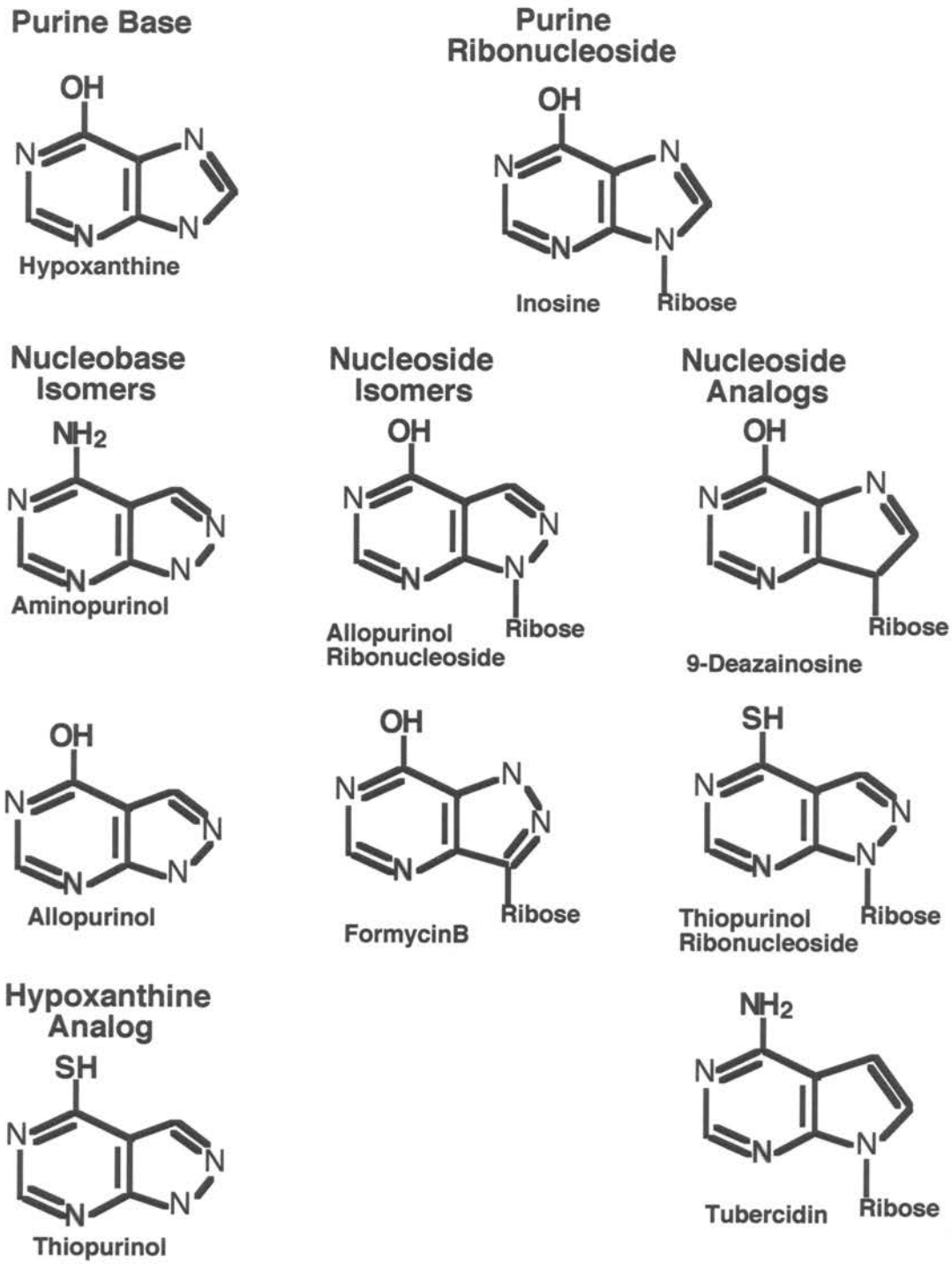


Figure 11. Analogs and isomers of purines and ribonucleosides

Genetic Reagent	Examples
Reporter genes	*LACZ, GUS, CAT, GFP*
Transient transfection	CAT, LACZ, GUS
Stable transfection and markers for positive selection	*NEO, HYG, PHLEO, PAC, SAT, BSD, NAGT, DHFR-TS*
Negative selection	*TK, CD*
Expression vectors and circular episomes	pX series
Chromosomally integrated	pIR1SAT, pSSU-int
Regulatable (inducible)	*Tet* repressor system
Gene arrays	*GP63, CYPB, a-Tub*
Transposon mutagenesis *in vivo* *in vitro*	 *mariner* *mariner*, Ty1
Loss of heterozygosity (LOH)	*DHFR-TS, HGPRT, APRT*
Functional genetic rescue	*LPG* biosynthetic genes
Artificial chromosomes (LACs)	*DHFR-TS* based on LAC
RNA interference (RNAi)	Not in *Leishmania* to date, (many successes in trypanosomes)
Selection from (19)	

Table 4. Genetic reagents that are available for use in *Leishmania*

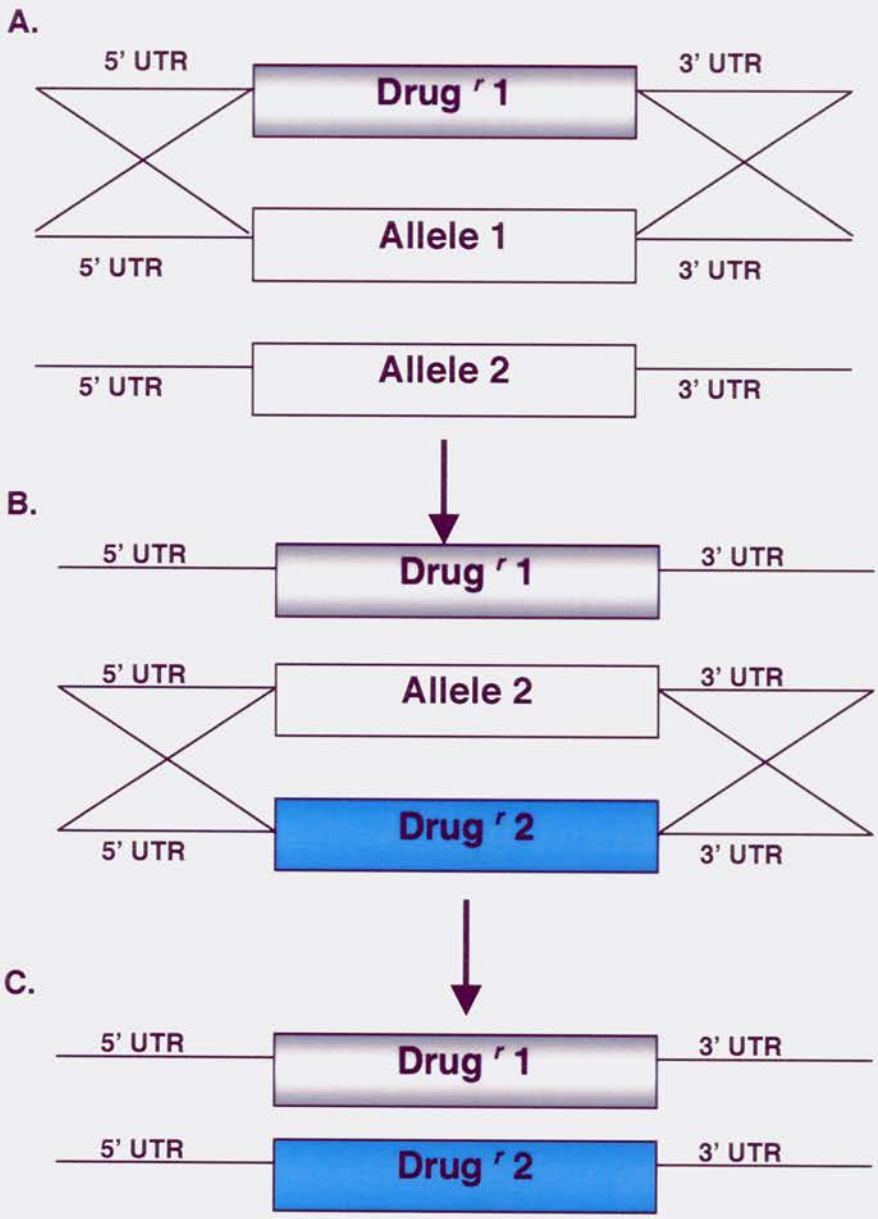


Figure 12. Targeted gene replacement as it occurs in *Leishmania*. The first replacement cassette is transfected in, homologous gene replacement occurs (A) and one allele of the gene of interest is replaced to generate heterozygotes (B). A second cassette is transfected in (B) and the second allele of the gene of interest is replaced, resulting in a homozygous knockout (C).

Cell Line
Wild Type
ak^{-}
aprt^{-}
Δhgprt
ak^{-}*/Δhgprt*
ak^{-}*/Δaprt*
Δhgprt/Δaprt
ak^{-}*/Δhgprt/Δaprt*

Figure 13. Cell lines generated in order to initiate a genetic analysis of the purine salvage pathway in *Leishmania donovani*.

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Chapter 2

Manuscript 1

Subcellular Localization of Adenine and Xanthine Phosphoribosyltransferases in *Leishmania donovani* *

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2.1. Abstract

The subcellular location of a protein is a critical factor in its physiological function and an important consideration in therapeutic paradigms that target the protein. Because *Leishmania donovani* cannot synthesize purine nucleotides *de novo,* they rely predominantly upon therapeutically germane phosphoribosyltransferase (PRT) enzymes, hypoxanthine-guanine PRT (HGPRT), adenine PRT (APRT), and xanthine PRT (XPRT), for purine acquisition from the host. Previous studies have shown that the *L. donovani* HGPRT is localized to the glycosome, a fuel-metabolizing microbody that is unique to kinetoplastid parasites (Shih *et al.*, *J. Biol. Chem.*, 273:1534-1541 (1998)). The sequences of the other two PRTs indicate that XPRT, but not APRT, possesses a COOH-terminal tripeptide that mediates protein targeting to the glycosome. To determine definitively the intracellular milieu of APRT and XPRT, polyclonal antibodies were raised to each recombinant protein. APRT and XPRT were then shown by immunofluorescence to be localized to the cytosol and glycosome, respectively. The glycosomal milieu for XPRT was also verified by immunoelectron microscopy. Amputation of the glycosomal targeting signal from XPRT resulted in protein mislocalization to the cytosol, but the cytosolic xprt was still functional with respect to purine salvage. These studies establish that APRT is cytosolic and XPRT, like the homologous HGPRT, is glycosomal and demonstrate that a mutant xprt protein that mislocalizes to the cytosol is still functional and supports parasite viability.

2.2. Introduction

Perhaps the most striking metabolic disparity between protozoan parasites and their eukaryotic hosts is the mechanisms by which they synthesize purine nucleotides. Whereas mammalian cells can synthesize purine nucleotides from amino acids and 1-carbon moieties, all protozoan parasites that have been studied to date are incapable of synthesizing the purine ring *de novo* [1]. Consequently, each parasite genus has evolved a unique complement of nutritionally indispensable purine salvage enzymes that enable the parasite to extract purines from their hosts. *Leishmania donovani* accommodates three biochemically and genetically distinct phosphoribosyltransferase (PRT) enzymes; hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), and xanthine phosphoribosyltransferase (XPRT), all of which are potentially attractive targets for drug design [1]. Each of the three *L. donovani PRT* genes has been isolated, knockout lines have been created by targeted gene replacement, and the recombinant proteins have been characterized with respect to substrate specificity and kinetic parameters and used as immunogens to generate monospecific antibodies [2-6].

Subcellular fractionation experiments conducted in the 1980s were the first to suggest that the HGPRTs of *L. mexicana* [7] and the related trypanosomatids *Trypanosoma brucei* [8] and *Trypanosoma cruzi* [9] were associated with the glycosome, a fuel metabolizing microbody that is unique to parasites of the Trypanosomatidae family [10, 11]. A glycosomal milieu for HGPRT in these parasites was further supported by the COOH-terminal

tripeptides predicted from the *L. donovani* [2] and *T. cruzi HGPRT* [12] gene sequences. These triads are consistent with the relatively degenerate COOH-terminal peroxisomal targeting signal – 1 (PTS-1) that mediates glycosomal targeting [13, 14]. A glycosomal location for the *L. donovani* [15] and *T. brucei* [16] HGPRT proteins was subsequently definitively established by immunocytochemical methods.

The deduced APRT and XPRT sequences from *L. donovani* indicate that XPRT, but not APRT, encompasses a PTS-1 [2, 6]. Because the subcellular milieu of a protein is an important determinant of its specialized function and can also impact drug targeting paradigms, we have implemented immunocytochemical methodologies in order to definitively establish the subcellular environments of these nutritionally important and therapeutically germane proteins. Using either deconvolution microscopy alone or a combination of deconvolution and immunoelectron microscopy, we have now established that the *L. donovani* APRT and XPRT enzymes are localized to the cytosol and glycosome, respectively. Furthermore, we have shown that targeting of XPRT to the glycosome is mediated by its PTS-1 and that a mislocalized xprt protein can still mediate xanthine uptake and salvage.

2.3. Materials and Methods

2.3.1. Parasites

L. donovani promastigotes were cultivated at 26°C in Dulbecco's modified Eagle - *Leishmania* medium and harvested for the fractionation and immunocytochemical experiments at a density of ~5 X 10^6 cells/ml. The wild type strain, designated DI700, was originally derived from the IS Sudanese clone. The construction and characterization of the *Δxprt* [6] and *Δaprt/Δhgprt* [4, 5] *L. donovani* lines has been described previously. The purine source for parasite cultivation was always 100 μM xanthine, except for the *Δxprt* strain, which was grown in 100 μM hypoxanthine.

2.3.2. Construction of XPRT expression vectors and transfection

The *L. donovani XPRT* was cloned and sequenced as described [6]. To create *XPRT* overexpressors within the *Δxprt* background, the *XPRT* open reading frame (ORF) was amplified by the polymerase chain reaction (PCR) on a PTC-100 (MJ Research Inc.) thermocycler using DI700 genomic DNA as a template, the Advantage™ – HF 2 PCR kit (BD Biosciences Clontech, Palo Alto, CA) for DNA amplification, and standard reaction conditions. The sense primer, 5'-CCCGGGATGCTACCAACCCACAGTTGTAAAG-3', was constructed to the first 25 nucleotides of the *XPRT* ORF and was preceded by a 6 nucleotide leader encompassing a *Sma*I site (underlined). The antisense primer, 5'-GGATCC**TCA**GAGCTTGGCAGGGTAACG-3', was synthesized to the last 18

nucleotides of the *XPRT* ORF and the stop codon (boldface type) and contained a 6 nucleotide leader with a *Bam*HI restriction site. To generate an xprt in which the PTS-1 signal (AKL) is deleted, the mutant *xprt* ORF was amplified using the same sense primer as above and an antisense primer, 5'-GGATCCCACTCCGGTGGTGTGACGTCGAGC**TCA**AGGGTAACGGGTGGCCTCC-3', constructed to amino acids 233-238 of the wild type XPRT (double underlined) and preceded by an artificially introduced in-frame stop codon (boldface type) that results in the elimination of the last 3 codons of XPRT. This antisense primer begins with a *Bam*HI site to enable cloning (underlined) followed by 24 nucleotides of 3' flanking residues just downstream from the stop codon of the wild type *XPRT.* The amplified PCR products were then digested with *Sma*I and *Bam*HI and subcloned into the matching sites within pXG-BSD [17], a leishmanial expression vector encoding the blasticidin S deaminase gene [18] that confers resistance to the antibiotic blasticidin. Both the wild type and COOH-terminal deletion constructs, designated pXG-BSD-*XPRT* and pXG-BSD-*xprtΔAKL*, respectively, were verified by nucleotide sequencing and subsequently transfected into *Δxprt* parasites by standard electroporation conditions for *Leishmania* [19, 20]. Transfected parasites were then selected in 25 μg/ml blasticidin. Transfectants harboring episomes encompassing the wild type and COOH-terminal deletion constructs were designated *Δxprt*[*pXPRT*] and *Δxprt*[*pxprtΔAKL*], respectively, according the generally accepted genetic nomenclature for *Leishmania* [21].

2.3.3. Xanthine uptake and salvage

The capacities of DI700, *Δxprt, Δxprt*[*pXPRT*] and *Δxprt*[*pxprt*ΔAKL] parasites to take up 1.8 μM xanthine (54 mCi/mmol) were performed as reported [6]. The abilities of these same strains to grow in the presence of xanthine as a purine source were also assessed as described [6]. Parasites in the growth assays were enumerated on a hemocytometer.

2.3.4. Antibody purification

Recombinant APRT [3] and XPRT [6] were purified from *E. coli* lysates using previously reported protocols. Both recombinant proteins were bound to affi-10 gel (BioRad) according to the manufacturer's protocol. The affinity matrices to which APRT and XPRT were bound were then used for affinity purification of APRT and XPRT antibodies from crude rabbit polyclonal antisera, respectively. Antibody titers were determined by ELISA and specificity confirmed via western blot analysis of DI700, *Δxprt,* and *Δaprt/Δhgprt L. donovani* lysates [3, 6]. ELISAs and Western blot analysis were performed using standard protocols.

2.3.5. Immunofluorescence

L. donovani promastigotes at a density of ~5 X 10^6/ml were pelleted by centrifugation at 3,000 X g for 10 min, rinsed with phosphate buffered saline (PBS) and affixed either to coverslips or to 4-well chamber slides (Nalge Nunc. International, Rochester, NY) that had been pretreated with poly-L-lysine. Cells

were fixed in a PBS solution containing 4% paraformaldehyde and 0.1% gluteraldehyde for 30 min at room temperature. Coverslips and chamber slides were rinsed once with PBS to remove fixative and incubated in a blocking solution consisting of PBS supplemented with 2% or 5% goat serum, 0.1% Tween-20, and 0.1% Triton X-100, which also permeablized the parasites, for 1 h at room temperature. Purified APRT antibodies were diluted 1:50 with blocking solution and incubated with the coverslips or chamber slides to which DI700 and *Δaprt/Δhgprt* cells were affixed. Purified XPRT antibodies were diluted 1:200 with blocking solution and incubated with DI700 and Δ*xprt* parasites. *L. mexicana* GAPDH antibodies were diluted 1:500 in blocking solution and applied to the DI700, *Δaprt/Δhgprt* and *Δxprt* cells to confirm that the glycosomes remained intact during cell preparation.

After incubation of fixed parasites with primary antibody for 1 h, all coverslips were rinsed 6 times for 5 min with a PBS wash solution containing 0.1% Tween-20. Fixed cells were next incubated with secondary anti-rabbit antibody conjugated to Texas Red dye (Molecular Probes) that had been diluted 1:1,000 in blocking buffer. The incubation and all subsequent steps were carried out in the dark. Secondary antibody was removed by soaking coverslips six times for five min in wash buffer and incubating in 3,3'-diohexyloxocarbocyanine iodide, a fluoroscein lipophilic dye, for 2 min. Coverslips were washed in PBS and affixed to slides with a solution consisting of 50 mM Tris, pH 8.0, 8% n-propylgalate (Sigma Chemical Co.), and 90% glycerol. Images for Figs. 2 and 3 were obtained by Ms. Aurelie Snyder of the OHSU-MMI Research Core Facility

at OHSU (http://www.ohsu.edu/core) with the Applied Precision Deltavision® image restoration system. Deconvolution was performed using the iterative constrained algorithm of Agard *et al.* [23] and additional image processing performed on an SGI Octane workstation. Images for Fig. 5 were taken on a Zeiss Axiovert 200 inverted microscope and deconvolution carried out using Axiovision 3.1 software (Carl Zeiss Optical, Chesterfield, VA). The mounting medium in Fig. 5 contained 0.75 μg ml^{-1} 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

2.3.6. Immunoelectron microscopy

50 ml of mid log stage DI700 and *Δxprt* cells were subjected to cryo-immunoelectron microscopy according to established protocols [15]. Mr. Jaime Dante of the Washington University Molecular Microbiology Imaging Facility performed the cryo-immunoelectron microscopy. Cells were pelleted, fixed, and rapidly frozen in liquid nitrogen and cryosectioned. Sections were collected on carbon-Formvar-coaged grids, blocked, and then probed with the affinity-purified XPRT antibodies, followed by incubation with an antirabbit secondary antibody conjugated to 15 nm gold particles. Cell sections were imaged on a Zeiss 902 transmission electron microscope by Dr. Wandy Beatty of the Washington University Molecular Microbiology Imaging Facility.

2.4. Results

2.4.1. Localization of APRT and XPRT by immunocytochemistry

To determine the subcellular compartment in which APRT and XPRT are found in *L. donovani* promastigotes, the enzymes were localized by immunocytochemical methods. Immunofluorescence analysis of APRT stained with Texas Red-conjugated secondary antibody revealed a diffuse staining pattern in wild type parasites (Fig. 1A). No staining was observed in wild type parasites incubated with APRT antibody that had been pretreated with excess recombinant *L. donovani* APRT (Fig. 1B) or in a *Δaprt* knockout line (Fig. 1C). The lack of staining within the *Δaprt* background could not be ascribed to an unforeseen absence of glycosomes, since incubation of the genetically manipulated cells with specific antibodies raised to the *L. mexicana* GAPDH, a known glycosomal marker [22], resulted in a distinct punctate pattern indicative of glycosomal localization (Fig. 1D). All parasites in Fig. 1 have been costained with a fluorescein lipid dye to simplify visualization. A cytosolic location for APRT could not be confirmed by immunoelectron microscopy, because the APRT antibody did not recognize the APRT protein on appropriately prepared cells.

The subcellular compartment in which XPRT is located was also assessed by immunofluorescence. As shown in Fig. 2A, wild type cells stained with Texas Red-conjugated secondary antibodies revealed a distinct punctate pattern of staining for XPRT that was not observed when antibody was pretreated with a surplus of protein (Fig. 2B) or with *Δxprt* parasites (Fig. 2C). The *Δxprt* parasites

were also stained with the leishmanial GAPDH antibodies resulting in a punctate pattern (Fig 2D). Immunoelectron microscopy was employed to authenticate glycosomal targeting of the XPRT. Gold particle labeling for XPRT was sequestered within the glycosomes in wild type parasites, and no other subcellular compartments were labeled to a significiant degree (Fig. 3). No immunogold labeling was observed with *Δxprt* parasites (Fig. 3B).

2.4.2. The XPRT PTS-1 is necessary for glycosomal targeting but not for function

It has been determined previously that the PTS-1 of XPRT is essential for the association of the enzyme *in vitro* through protein-protein interactions with the *L. donovani* peroxin 5, a cytosolic receptor involved in sorting proteins for import into the glycosome [23]. To authenticate whether the putative PTS-1 was required for targeting XPRT to the glycosome *in vivo*, *Δxprt* parasites were transfected with either a wild type *XPRT* or a mutated *xprt* construct lacking the codons for the Ala-Lys-Leu PTS-1. Western blot analysis verified the presence of XPRT protein in both the *Δxprt*[*pXPRT*] and the *Δxprt*[*pxprtΔAKL*] transfectants (Fig. 4). Indirect immunofluorescence analysis of the XPRT in the *Δxprt*[*pXPRT*] line revealed the expected punctate staining of a wild type XPRT enzyme, whereas the staining pattern for the xprtΔAKL protein produced in the mutant *Δxprt*[*pxprtΔAKL*] transfectant was diffuse and similar to the staining pattern observed using APRT antibody (Fig. 1A and Fig. 5).

To evaluate whether the PTS-1 was essential for XPRT function in promastigotes, the ability of wild type, *Δxprt*, *Δxprt*[*pXPRT*], and *Δxprt*[*pxprtΔAKL*] to grow with xanthine as a purine source was compared. Whereas the *Δxprt* knockout could not grow in xanthine-containing DME-L, both *Δxprt*[*pXPRT*] and *Δxprt*[*pxprtΔAKL*] were able to proliferate on the purine nucleobase (Fig 7). Furthermore, the *Δxprt*[*pXPRT*] and *Δxprt*[*pxprtΔAKL*] transfectants were both able to incorporate xanthine from the culture medium to equivalent extents, although uptake by *Δxprt*[*pxprtΔAKL*] cells occurred at consistently lower rates than by wild type or *Δxprt*[*pXPRT*] parasites (Fig. 7).

2.5. Discussion

The inability of parasites to synthesize purine nucleotides from small molecule precursors and the reliance of these pathogens on purine acquisition from the host implies that components of the purine salvage pathway may be targets for inhibitory drug design. *Leishmania* accommodate 3 biochemically and genetically distinct PRT enzymes, HGPRT, APRT, and XPRT, all of which are capable of converting preformed purine bases to the nucleotide level. In these studies, the APRT and XPRT enzymes from *L. donovani* have been definitively localized to the parasite cytosol and glycosome, respectively using immunocytochemical methods. Specificity of the antibodies directed against the APRT and XPRT epitopes was established previously by western blotting [4, 6] and now by the ability of excess purified recombinant enzyme to block recognition of its cognate native counterpart and the failure of the antisera to recognize any protein in intact *Δaprt* or *Δxprt* parasites.

A glycosomal milieu was initially inferred for the *L. donovani* XPRT protein based on the predicted COOH-terminal tripeptide, Ala-Lys-Leu, a sequence compatible with the acceptable degeneracy of the PTS-1 that is recognized by the glycosomal import machinery of *Leishmania* and trypanosomes [13, 14, 23]. This PTS-1 is comprised of a first amino acid that is generally small and uncharged, a second amino acid that is usually basic and capable of forming hydrogen bonds, and a third that is hydrophobic. Thus, the COOH-terminal tripeptide of the *L. donovani* APRT, His-Pro-His, is a sequence that is incompatible with PTS-1 recognition criteria. Deletion of the PTS-1 from XPRT

eliminates the topogenic signal for glycosomal targeting, and the mutant xprt now mislocalizes to the cytosol. Because transfection of the *Δxprt* strain with pXG-BSD-*xprtΔAKL* restores the ability of the knockout to proliferate on xanthine as a purine source (Fig. 6), it is obvious that a glycosomal milieu for the enzyme is not imperative for xanthine salvage by the promastigote. However, xanthine incorporation by the *Δxprt*[*pxprtΔAKL*] line is significantly and consistently less efficient than xanthine uptake by either wild type or *Δxprt*[*pXPRT*] parasites (Fig. 7), indicating that mislocalization does seem to impair physiological activity, although it does not impact upon growth of the promastigote in culture. Thus, the ability to take up exogenous radiolabeled xanthine through XPRT activity is essential but not rate limiting for proliferation. The basis for the constraints on xanthine salvage capability by *Δxprt*[*pxprtΔAKL*] cells is unclear but may have to do with limitations on the rates by which XPRT products such as XMP and IMP can access subsequent metabolic machinery for nucleotide interconversion (see below).

Previous studies with *Leishmania* have ascertained that HGPRT, like XPRT, is localized predominantly, if not exclusively, in the glycosome. The leishmanial HGPRT cosediments with the glycosomal GAPDH (data not shown), and antibodies specific for the *L. donovani* HGPRT demonstrated glycosomal localization using indirect immunofluorescence and immunoelectron microscopy [15]. Moreover, the *L. donovani* HGPRT also accommodates a PTS-1, Ser-Lys-Val, which, when deleted, precludes glycosomal targeting in *Δhgprt* parasites transfected with a mutant *hgprt* expression construct lacking its PTS-1 [15].

The explanation for the glycosomal compartmentalization of the *L. donovani* XPRT and HGPRT enzymes, as well as the differential localization of the APRT, is unclear, as the mammalian PRTs are cytosolic. However, because glycosomes accommodate an assortment of fuel metabolizing enzymes that perform vital nutritional roles for the parasite and purine salvage is requisite for parasite survival, the restricted view of the glycosome as a fuel metabolizing organelle should be expanded to include other essential nutrients. It should be noted, however, that none of the PRTs by itself is crucial to the survival or normal proliferation of the promastigote stage of *Leishmania*, because *Δhgprt, Δxprt*, and *Δaprt* parasites are perfectly viable [4-6]. A glycosomal environment is also not necessary for promastigote viability because *Δhgprt* and *Δxprt* transfected with HGPRT and XPRT constructs, respectively, lacking their PTS-1, appear normal in all respects, although the mislocalized xprt does not seem to incorporate exogenous xanthine as efficiently as its wild type counterpart ([15] and Figs. 7 and 8). One practical hypothesis for the glycosomal compartmentalization of XPRT and HGPRT is access to substrate. However, purine bases must obligatorily be obtained from the extracellular milieu, whereas the 5-phosphoribosyl-1-pyrophosphate (PRPP) substrate is generated by PRPP synthetase, from ribose-5-phosphate and ATP. Although the location of the parasite PRPP synthetase is unknown, the enzyme terminates in a Arg-Asp-Ser sequence [24], an improbable candidate for a PTS-1 [13, 14].

The location of other purine salvage and interconversion enzymes in *Leishmania* has also not been conclusively evaluated. The Ala-Lys-Met COOH-

terminal tripeptide of the *L. donovani* IMP dehydrogenase [25] that initiates the metabolism of the IMP product of HGPRT and XPRT toward guanylate nucleotide synthesis is a likely PTS-1, as are the final 3 amino acids (Leu-Arg-Gln) of the functionally uncharacterized *L. major* adenylosuccinate synthetase (L. major database, contig 893447, gene L2464.06) that directs IMP to the adenylate branch of purine nucleotide synthesis. Other homologs of purine enzymes in the *L. major* database do not appear to accommodate PTS-1s but may still possess other glycosomal targeting signals [10,11]. If IMP dehydrogenase, adenylosuccinate synthetase, and other purine salvage enzymes are proven to be sequestered within the glycosome, the ability of *Δxprt*[*pxprtΔAKL*] parasites to grow with xanthine as the sole purine source implies that the glycosomal membrane is permeable to nucleoside monophosphates. Despite the lack of an unambiguous explanation for the unusual organellar distribution of purine salvage enzymes in *Leishmania* and related trypanosomatids, the clear-cut association of therapeutically germane purine salvage enzymes with the glycosome is noteworthy not only from a biological perspective but also from a drug development point of view, as drugs that target HGPRT or XPRT, such as the pyrazolopyrimdine nucleobase analogs [26, 27], must traverse the glycosomal membrane to exert their antiparasitic effects.

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2.6. Figures

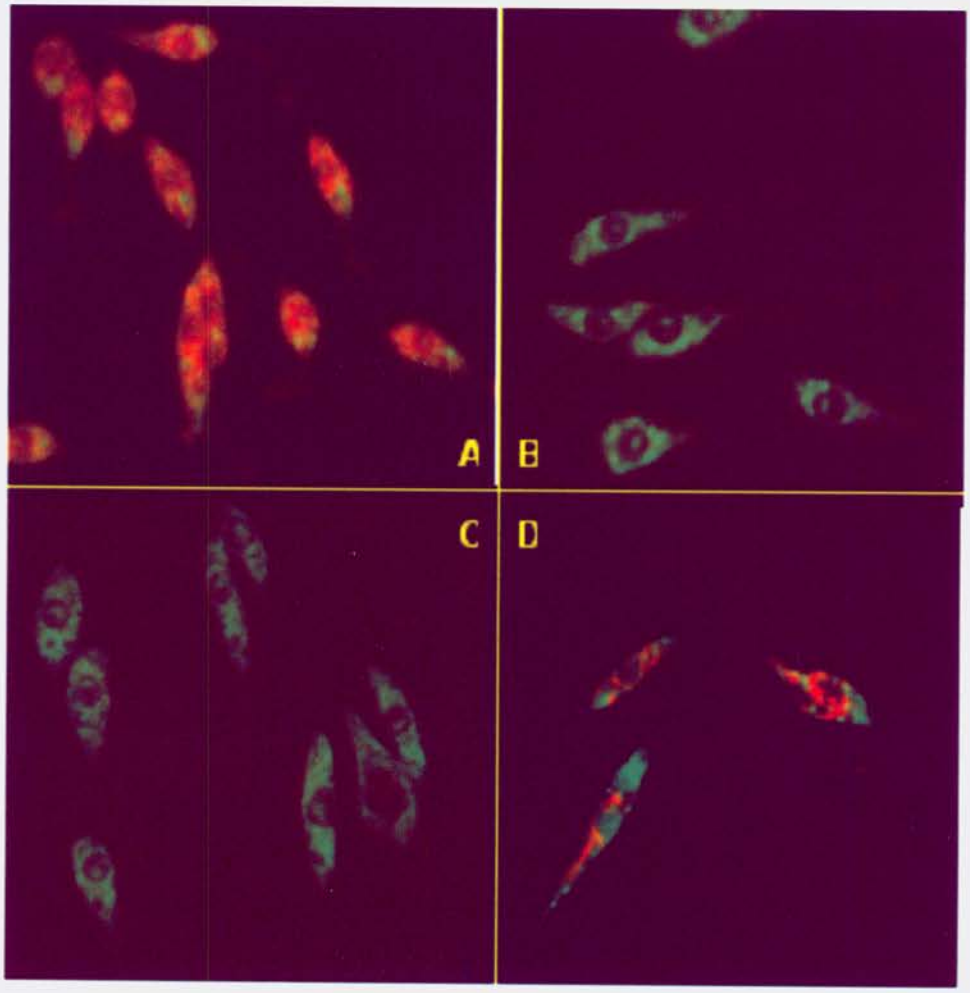

Figure 1. Deconvolved scanning micrographs of *L. donovani* promastigotes stained with APRT antibody. *L. donovani* promastigotes were visualized at 546 nm for the presence of APRT or GAPDH using a Texas Red-conjugated secondary antibody. (A), wild type parasites incubated with APRT antisera (B), wild type *L. donovani* probed with APRT antibodies that had been preincubated with excess pure recombinant APRT protein (C), *Δaprt/Δhgprt* parasites incubated with APRT antibodies, and (D), *Δaprt/Δhgprt* cells incubated with GAPDH antibodies to show that glycosomes are intact. Parasites were counterstained with a fluorescein lipid dye using illumination at 488 nm.

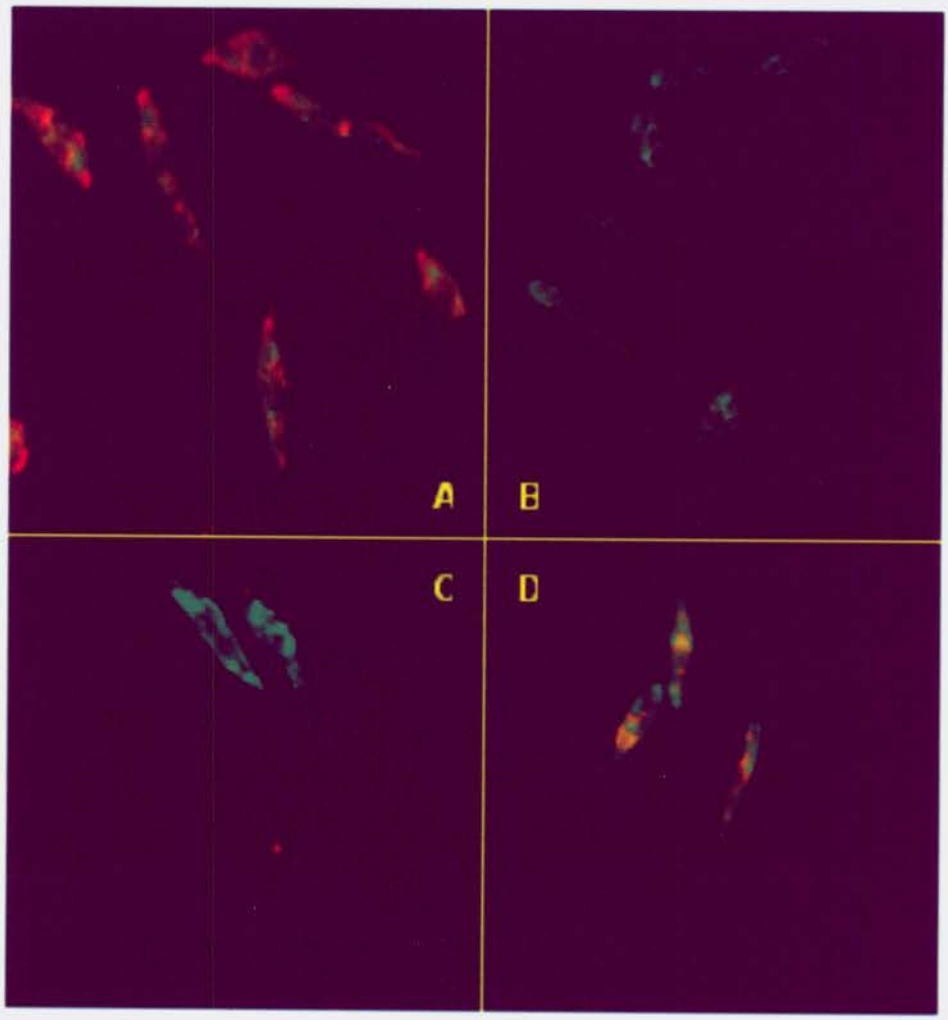

Figure 2. Double label deconvolution microscopy using XPRT antisera. Wild type (A-B) and *Δxprt* (C-D) *L. donovani* were incubated with purified XPRT antibodies (A,C), XPRT antibodies that had been incubated with excess recombinant XPRT protein (B), or GAPDH antibodies and visualized at 546 nm as described in the legend to Fig. 1. Parasites were visualized with the fluoroscein lipid dye at 488 nm.

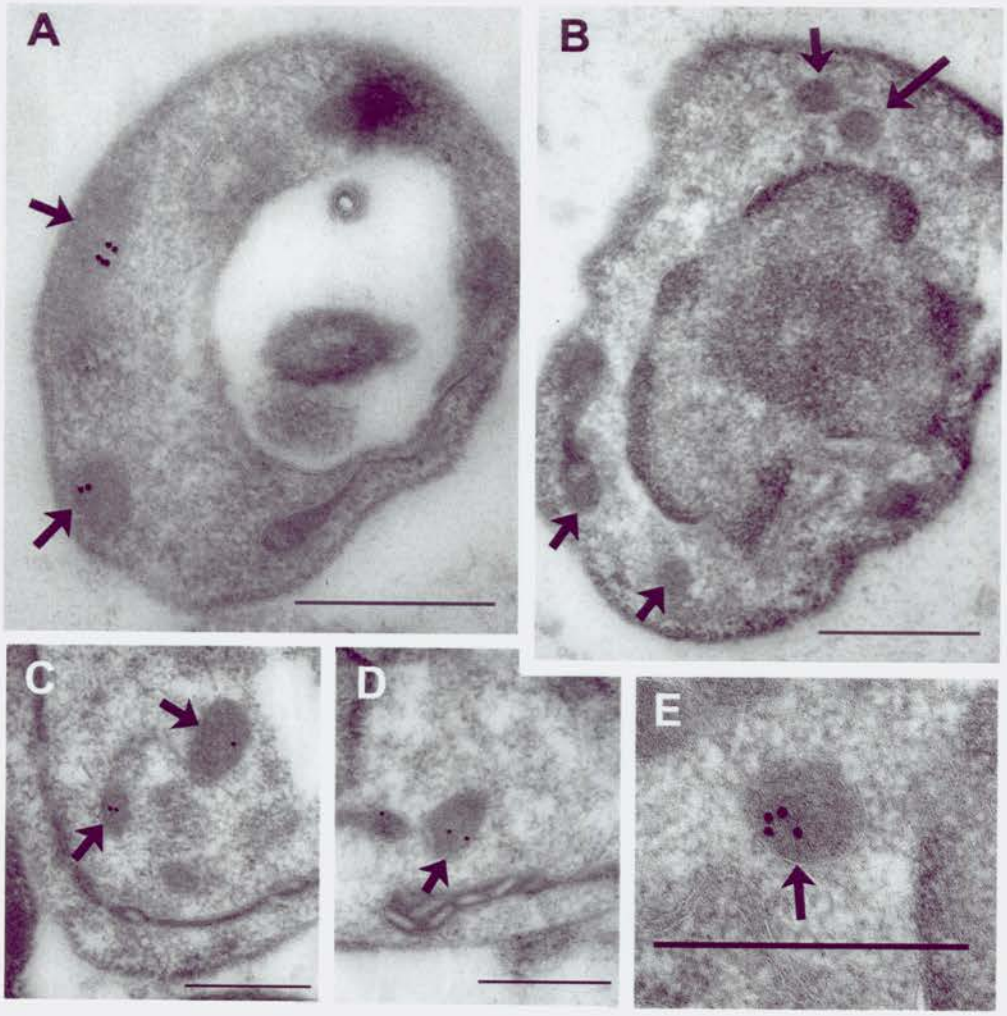

Figure 3. Transmission electron micrographs of ultrathin cryosections of *L. donovani* immunolabeled with anti-XPRT antibodies. Wild type (A,C-E) and *Δxprt* (B) *L. donovani* were probed with purified antibodies raised to XPRT and visualized with a secondary antibody conjugated to 15nm gold particles at 40,000 X magnification. Glycosomes are indicated by arrows. Scale bar equals 20nm.

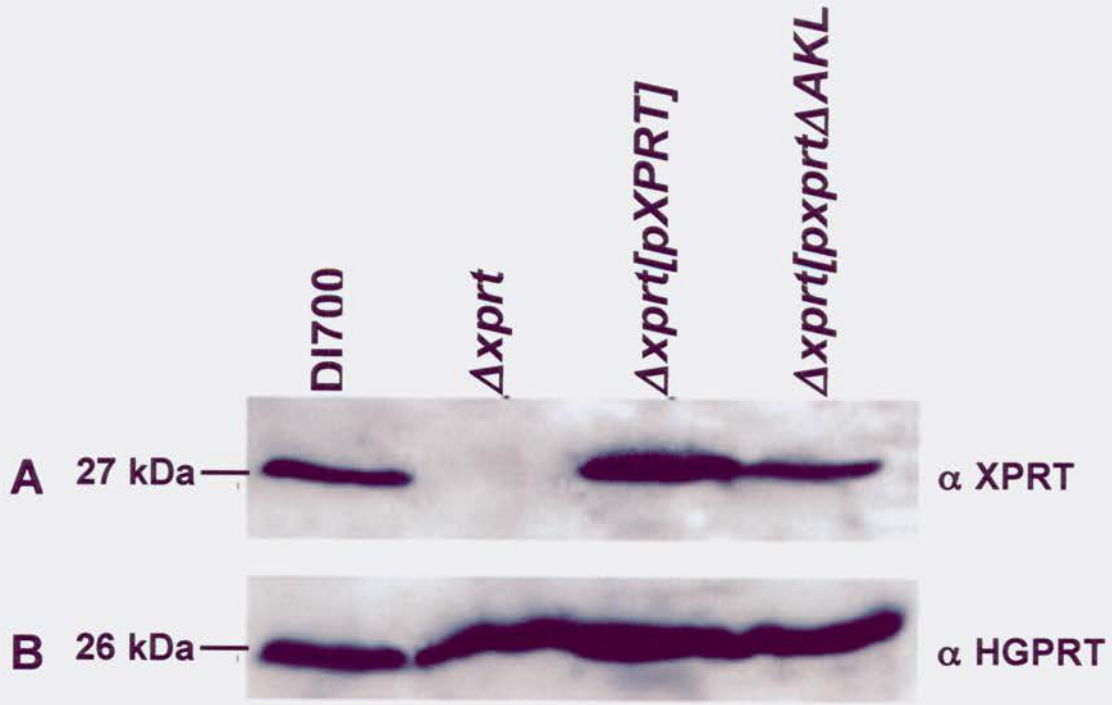

Figure 4. Immunoblot analysis of XPRT expression in wildtype, *Δxprt*, and transfectant cell lines. (A), lysates of exponentially wild type, *Δxprt, Δxprt[pXPRT]* and *Δxprt[pxprtΔAKL]* parasites were analyzed by Western blot analysis using the anti-XPRT polyclonal antisera (α XPRT), (B), the amount of cell lysate loaded onto each lane of the gel was normalized using rabbit antisera raised against the *L. donovani* HGPRT (α HGPRT).

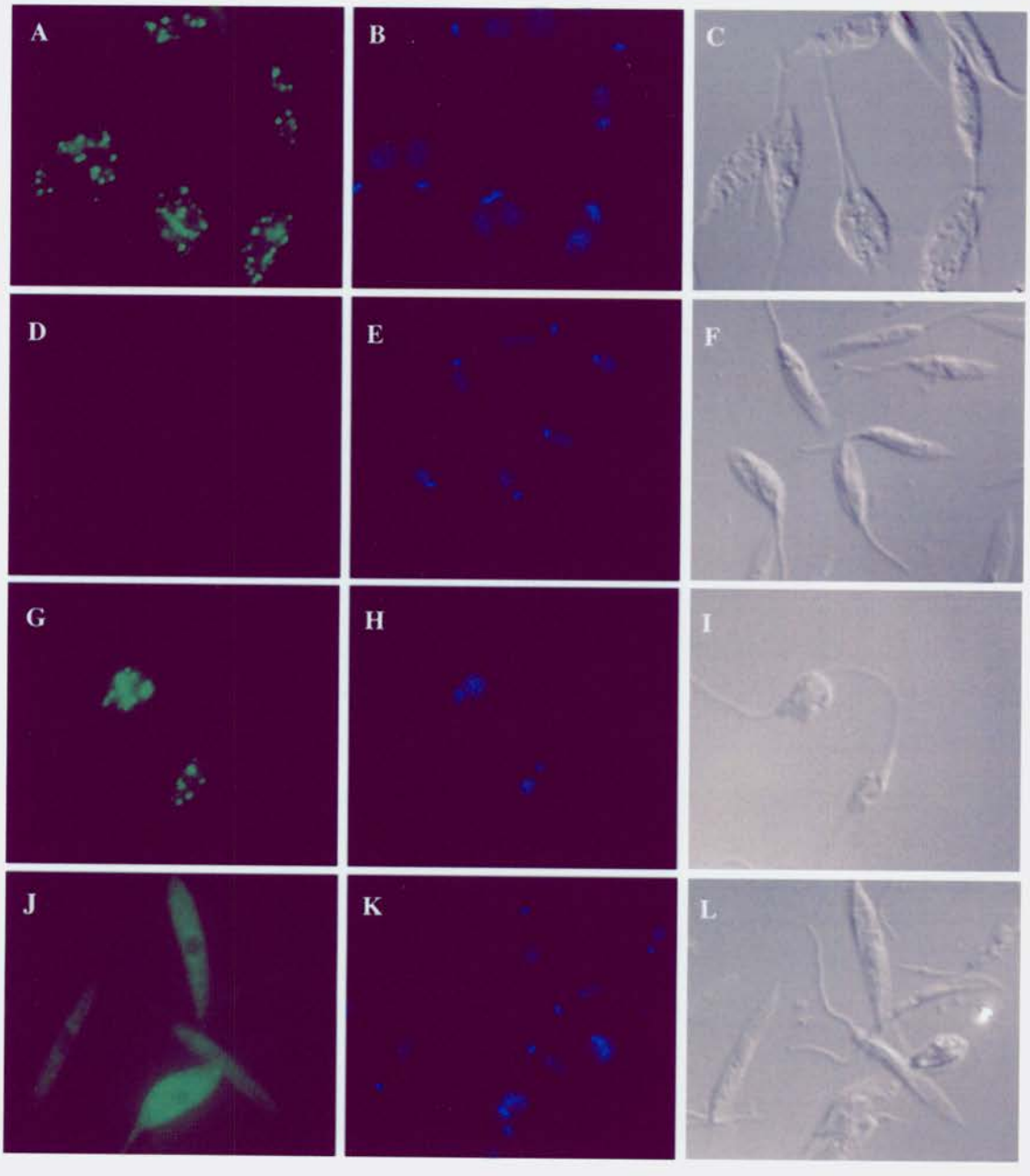

Figure 5. Localization of XPRT is mediated by its PTS-1. Deconvolution microscopy was performed with wild type DI700 (panels A - C), *Δxprt* knockouts (panels D - F), and in *Δxprt*[*pXPRT*] (panels G - I) and *Δxprt*[*pxprtΔAKL*] (panels J - L) parasites using anti-XPRT antisera (panels A, D, G, and J) using

parameters and conditions essentially identical to those described in the legend to Fig. 1. Panels B, E, H, and K are parasites stained with 4',6-diamidino-2-phenylindole for DNA visualization. Panels C, F, I, and L are phase contrast images of the stained parasites.

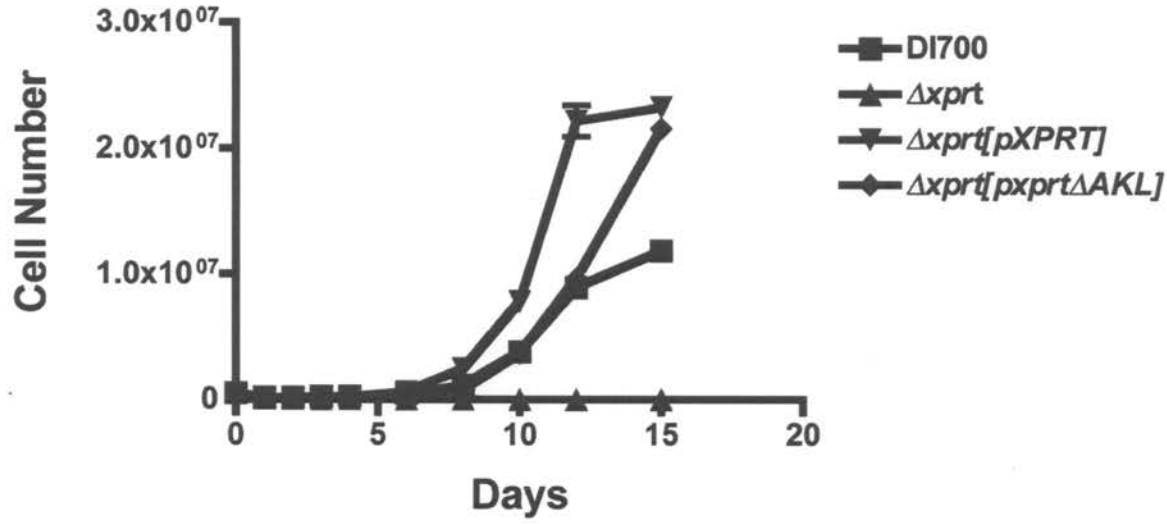

Figure 6. Growth rates of wild type, *Δxprt, Δxprt*[*pXPRT*], and *Δxprt*[*pxprtΔAKL]* parasites in xanthine. The rate of growth of wild type, *Δxprt*, *Δxprt*[*pXPRT*], and *Δxprt*[*pxprtΔAKL*] parasites in DME-L with xanthine as the sole source of purine was conducted over 15 days. Parasites were enumerated on a hemocytometer, and data points are the averages ± standard errors of three independent samples.

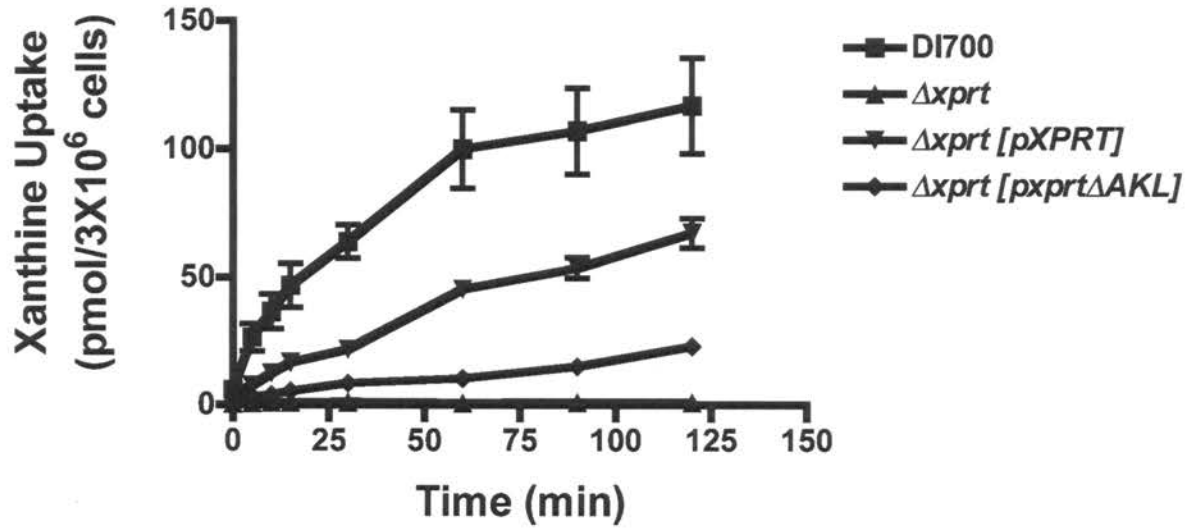

Figure 7. Xanthine uptake by *Δxprt*[*pXPRT*], and *Δxprt*[*pxprtΔAK*L] parasites. The capacities of DI700, *Δxprt*, *Δxprt*[*pXPRT*], and *Δxprt*[*pxprtΔAKL*] parasites to convert 1.8 μM [^{14}C]xanthine to the nucleotide level were ascertained as described by Hwang and Ullman [5]. Each time point reflects the amount of radiolabel in phosphorylated metabolites incorporated by 3 X 10^8 parasites that had been purine-starved for 48 h and was performed in triplicate.

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Chapter 3

Manuscript 2*

Leishmania donovani Mutants Deficient in Purine Phosphoribosyltransferases are Viable and Infectious

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Abbreviations: PRT, phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; (APRT), adenine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AK, adenosine kinase; dCF, 2'-deoxycoformycin; DME-L, Dulbecco's modified Eagle-*Leishmania*; PCR, polymerase chain reaction; UTRs, untranslated regions; G418, Geneticin; PBS, phosphate buffered saline; AAH, adenine aminohydrolase.

3.1. Abstract

Leishmania species express three phosphoribosyltransferase enzymes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), and xanthine phosphoribosyltransferase (XPRT), which enable this genus to acquire purine nutrients from their hosts. To test whether any of these enzymes is essential for viability, transformation into amastigotes, and infectivity and proliferation within mammalian macrophages, *Δhgprt*, *Δaprt*, and *Δxprt* null mutants were created by targeted gene replacement within a virulent background of *L. donovani*. Each of the three knockout strains was viable as promastigotes and axenic amastigotes and capable of maintaining an infection in bone marrow-derived murine macrophages. These data support the hypothesis that none of the three phosphoribosyltransferases is essential for purine salvage or viability by itself and that purine salvage occurs through multiple anabolic routes in both parasite life cycle stages. In addition these studies revealed the presence of an adenine aminohydrolase enzyme in *L. donovani* amastigotes, an activity previously thought to be restricted to promastigotes.

Key Words: Purines, phosphoribosyltransferases, metabolism, infectivity.

3.2. Introduction

Leishmania is a digenetic parasite that exists as the flagellated, extracellular promastigote in the insect vector host and as an immotile, intracellular amastigote within macrophages and reticuloendothelial cells of the infected mammalian host. Like all protozoan parasites studied to date, *Leishmania spp.* lack the capacity to synthesize the purine ring *de novo* and consequently have evolved a series of unique purine salvage enzymes that are essential for scavenging purines from their hosts (1, 2). *Leishmania* express three distinct phosphoribosyltransferase (PRT) enzymes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), and xanthine phosphoribosyltransferase (XPRT), that convert purine bases to the nucleotide level, as well as an adenosine kinase (AK) that phosphorylates adenosine directly to AMP (3). The creation of *Δhgprt*, *Δaprt*, and *Δxprt* knockouts in *L. donovani* by targeted gene replacement approaches and the isolation of both $aprt^-$ and ak^- mutants after chemical mutagenesis have demonstrated that none of the salvage enzymes is essential by itself for viability of the insect vector form of the parasite (3-6). However, these mutants were all generated in an avirulent *L. donovani* background, and therefore the requirement of these proteins in infection of the mammalian host could not be ascertained.

To determine whether HGPRT, APRT, and XPRT are essential for purine nutrition and parasite viability within the infectious form of the parasite, *Δhgprt*,

Δaprt, and *Δxprt* null mutants were re-created within an *L. donovani* strain, LdBob (7), that retains its ability to transform to the amastigote and infect macrophages. All three knockouts were capable of transforming into axenic amastigotes and of sustaining an infection in bone marrow-derived murine macrophages. These data demonstrate that neither HGPRT, APRT, nor XPRT is essential by itself to either amastigotes or promastigotes and imply that multiple routes of purine acquisition exist for both life cycle stages of the parasite. These findings impact theoretical therapeutic paradigms that might target purine salvage in *Leishmania*.

3.3. Materials and methods

3.3.1. Chemicals and reagents

[8-^{14}C]adenine (50 mCi/mmol), [8-^{14}C]adenosine (53 mCi/mmol), [8-^{14}C]guanine (55 mCi/mmol), [8-^{14}C]guanosine (50 mCi/mmol), [8-^{14}C]hypoxanthine (51 mCi/mmol), [8-^{14}C]inosine (52 mCi/mmol), and [8-^{14}C]xanthine (53 mCi/mmol) were obtained from Moravek Biochemicals (Brea, CA), while [^{32}P]dCTP was bought from MP Biomedicals (Irvine, CA). 2'-deoxycoformycin (dCF) was acquired from the National Cancer Institute (Bethesda, MD). All restriction and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA), and all other chemicals and reagents were of the highest quality commercially available unless otherwise specified.

3.3.2 Parasite cell culture

The *L. donovani* strain LdBob (7) was obtained from Dr. Stephen Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultured at 26 °C in purine-replete M199-based medium (7) or in Dulbecco's modified Eagle-*Leishmania* (DME-L) medium (8) that lacked bovine serum albumin and was supplemented with 10% dialyzed fetal bovine serum, 1 mM glutamine, 1 X RPMI 1640 vitamin mix, 10 μM folate, and 100 μM purine. Axenic amastigotes were cultured at 37 °C as described (7).

3.3.3. DNA manipulations

Isolation of genomic DNA and Southern blotting were performed by conventional protocols (9). Hybridization probes were the full length coding sequences for *HGPRT*, *APRT*, and *XPRT*, all of which were generated by the polymerase chain reaction (PCR) and gel-purified using a QIAEX II kit (Qiagen, Valencia, CA). The plasmids from which the hybridization probes were amplified harbored the full length genes that had been cloned into the TOPO-TA vector (Invitrogen Corp., Carlsbad, CA).

3.3.4. Targeting and episomal constructs

The flanking regions from the *HGPRT*, *APRT*, and *XPRT* loci and the oligonucleotides used for their amplification by the polymerase chain reaction (PCR) have been described (4, 5, 10). The construction and authentication of the pX63-NEO-*Δhgprt*, pX63-HYG-*Δhgprt*, pX63-NEO-*Δaprt*, pX63-NEO-*Δxprt*, and pX63-HYG-*Δxprt* targeting vectors employed in the previous allelic replacements of the *HGPRT*, *APRT*, and *XPRT* loci have also been described (4, 5, 10). The pX63-PHLEO-*Δxprt* and pX63-PAC-*Δxprt* replacement plasmids used to generate the *Δxprt* line were generated by excising the 5' and 3' untranslated regions (UTRs) of *XPRT* from pX63-HYG-*Δxprt* and inserting them into the appropriate sites within pX63-PHLEO and pX63-PAC, two leishmanial expression plasmids (11).

To generate episomal constructs of *HGPRT*, *APRT*, and *XPRT*, the three genes were amplified by PCR from genomic DNA, sequenced to ensure fidelity, and inserted into the SmaI-BamHI restriction sites within the pXG-BSD blasticidin resistance expression plasmid generously provided by Dr. Stephen Beverley (Washington University, St. Louis, MO). The complementation vectors were designated pXG-BSD-*HGPRT*, pXG-BSD-*APRT,* and pXG-BSD-*XPRT*, respectively.

3.3.5. Creation of null mutants and complemented strains

5 X 10^7 LdBob promastigotes were transfected using standardized electroporation conditions for LdBob (7). Electroporated parasites were maintained for 24 hr in liquid medium prior to plating on semi-solid M199-based growth medium supplemented with the appropriate purine at a concentration of 100 μM and containing the appropriate selective agents. Drug concentrations employed for the genetic manipulations were 20 μg/ml Geneticin (G418), 50 μg/mL hygromycin, 50 μg/ml phleomycin, 20 μg/ml puromycin, and 20 μg/ml blasticidin.

pX63-NEO-*Δhgprt*, pX63-HYG-*Δhgprt*, pX63-NEO-*Δaprt*, pX63-PHLEO-*Δxprt* and pX63-*PAC-Δxprt* were all linearized by digestion with HindIII and BglII and the fragments harboring the drug resistance cassette and the UTRs of the genes to be targeted were gel purified just prior to electroporation. The linear

drug resistance cassettes were designated by the name of the plasmid minus the initial letter, e.g., X63-HYG-*Δhgprt* from pX63-HYG-*Δhgprt*.

The schemes for generating the three knockout lines were as follows. A *HGPRT/hgprt* heterozygote was generated after transfecting wild type LdBob with X63-NEO-*Δhgprt*, and the *Δhgprt* null mutant was isolated after transfection of the *HGPRT/hgprt* heterozygote with X63-HYG-*Δhgprt*. The *APRT/aprt* heterozygote was isolated after transfection with pX63-NEO-*Δaprt*, and the *Δaprt* was obtained by selection for loss-of-heterozygosity on plates containing 100 μM 4-aminopyrazolopyrimidine, a cytotoxic substrate of APRT (4, 12). Finally, the *XPRT/xprt* heterozygote was generated after transfection of wild type parasites with X63-PHLEO-*Δxprt* and the *Δxprt* knockout obtained after transfection of the heterozygote with X63-PAC-*Δxprt*. The genotypes of all heterozygous and knockout strains were confirmed by Southern blot analysis.

For the macrophage infection experiments, derivatives of the *Δhgprt*, *Δaprt*, and *Δxprt* null mutants were constructed that harbored episomal copies of the gene that had been eliminated by targeted gene replacement. *Δhgprt*, *Δaprt*, and *Δxprt* promastigotes were transfected with pXG-BSD-*HGPRT*, pXG-BSD-*APRT*, and pXG-BSD-*XPRT*, respectively, to create the episomally complemented lines *Δhgprt*[p*HGPRT*], *Δaprt*[p*APRT*], and *Δxprt*[p*XPRT*].

3.3.6. Western blotting

Immunoblotting was carried out by standard protocols (9). Monospecific antibodies to purified recombinant *L. donovani* HGPRT, APRT, and XPRT proteins have been described previously (5, 13, 14). Mouse monoclonal antibody against the amastigote-specific A2 protein (15) was generously provided by Dr. Greg Matlashewski of McGill University Faculty of Medicine.

3.3.7. Enzyme assays

Cell-free lysates were prepared by sonication of 2 X 10^9 parasites that had been washed in phosphate buffered saline (PBS) and resuspended in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM phosphoribosylpyrophosphate, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Radiolabel incorporation assays were carried out with promastigote extracts as described (6), while amastigote enzyme assays were conducted with lysates that were not fractionated by centrifugation.

3.3.8. Purine metabolism in intact cells

The capacities of live cells to metabolize various purines to phosphorylated products were measured at ambient temperature using the DE-81 filter disk method (6). Each measurement was obtained with 10^7 intact parasites that had been resuspended in modified DME-L lacking BSA, FBS and hemin at a concentration of 10^8 cells per ml. Each aliquot was washed once in 1.0 ml of PBS, and the cells lysed in 50 µl of 1% Triton X-100. The extracts were

applied to DE-81 filter disks and the disks were washed as described [6]. Disks were air-dried, and radioactivity incorporated was quantified by scintillation counting.

3.3.9. Growth phenotypes

To evaluate the capabilities of the *Δhgprt*, *Δaprt*, and *Δxprt* knockouts to proliferate in different purines, wild type and mutant parasites were washed several times with PBS and resuspended in purine deplete DME-L-based medium that lacked BSA and that was supplemented with 10% dialyzed FBS. 1.0 ml aliquots of cells were seeded at a concentration of 5 X 10^4 parasites/ml and incubated for 5-7 days in this DME-L based medium to which various purine supplements were added to a final concentration of 100 μM. Amastigotes were seeded at a density of 5 X 10^3 parasites/ml in amastigote media that contained 20% FBS. Cells were enumerated visually by hemacytometer.

3.3.10. Macrophage infectivity assays

2 X 10^6 wild type or mutant promastigotes were washed two times in PBS and placed with either 2 X 10^5 bone marrow-derived macrophages from Balb/c mice or cultured J774 murine macrophages (ATCC, Manassas, VA) into 4-well Lab-TekII Chamber Slides (Nalge Nunc International Corp., Naperville, IL) containing 1.0 ml DME-M medium supplemented with 4mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% fetal bovine serum and incubated

at 37 °C in a humidified 5% CO_2 incubator. After 16 hr, adherent macrophages were rinsed ten times in PBS to eliminate remaining extracellular promastigotes after which fresh growth medium was added and then again 24 hr later. After an additional 24 hr incubation, the chambers were washed three times with PBS and macrophages stained using the Diff-Quik kit (International Medical Equipment Inc., San Marcos, CA). Parasites were detected on a Zeiss Axiovert 200M scope (Carl Zeiss Microimaging, Thornwood, NY) using a 60X oil immersion lens. Pictures were taken with an AxioCam MRm camera (Carl Zeiss Microimaging) and parasites enumerated. Color photographs of parasitized macrophages were detected on a Zeiss Axiophot microscope using 40X oil immersion and photographed with a Leica DC 300 camera (Leica Camera AG, Solms, Germany).

3.4. Results

3.4.1. Confirmation of the knockout genotypes

Δhgprt, *Δaprt*, and *Δxprt* parasites were created by targeted gene replacement within the infectious LdBob background. To confirm the genetic lesions, Southern blotting of genomic DNA from wild type, heterozygous, and homozygous knockout parasites was performed to demonstrate the homologous gene replacement events in the genetically manipulated strains (Fig. 1). Southern blot analysis using the *HGPRT*, *APRT*, and *XPRT* open reading frames as hybridization probes divulged the loss of both wild type alleles in each of the null mutant lines, whereas the *HGPRT/hgprt*, *APRT/aprt*, and *XPRT/xprt* heterozygotes retained at least one wild type copy of the gene that hybridized to the radiolabelled probe. The hybridization signals corresponded to the size of the restriction fragments predicted from the sequence of the *HGPRT*, *APRT*, and *XPRT* ORFs and adjacent 5' and 3' UTRs (4, 5). The nature of the replacements in the heterozygotes was also confirmed by probing with either the 5' or 3' UTR of the corresponding gene, an analysis that was performed after clonal isolates of the heterozygotes were initially obtained (data not shown).

3.4.2. Western blot analysis

Western blot analysis of wild type and mutant parasite cell-free extracts confirmed the absence of HGPRT, APRT, and XPRT protein in strains in which

the corresponding gene had been eliminated (Fig. 2). HGPRT, APRT, and XPRT protein was detected, as expected, in extracts of the *HGPRT/hgprt*, *APRT/aprt,* and *XPRT/xprt* heterozygotes, respectively, as well as the wild type progenitor (Fig. 2).

3.4.3. Enzyme assays in promastigote extracts

The abilities of wild type and null mutant promastigote lysates to phosphoribosylate radiolabelled purine to the nucleotide level were then assessed over a 15 min time course (Fig. 3). No hypoxanthine incorporation was detected in *Δhgprt* lysates, whereas the *HGPRT/hgprt* parasites incorporated hypoxanthine at ~50% the rate of that of the wild type parent (Fig 3A). Conversely, the wild type, *APRT/aprt*, and *Δaprt* extracts all incorporated adenine at similar rates. Because *L. donovani* promastigotes are known to express an adenine aminohydrolase (AAH) activity (2, 16) that is capable of converting adenine to hypoxanthine, a substrate for both HGPRT and XPRT (5, 14), [^{14}C]adenine incorporation into *Δaprt* parasite extracts was evaluated in the presence of 20 μM dCF, an inhibitor of AAH (16). The addition of 20 μM dCF to *Δaprt* extracts obliterated [^{14}C]adenine incorporation into nucleotides but had essentially no effect on the ability of the wild type parasite lysate to convert the purine to anionic metabolites. Xanthine phosphoribosylation to XMP in wild type, *XPRT/xprt*, and *Δxprt* extracts could not be determined for technical reasons.

3.4.4. Purine metabolism in intact promastigotes

The ability of intact wild type and knockout parasites to metabolize exogenous purine nucleobase into nucleotides was also gauged. Wild type parasites were capable of efficiently converting [^{14}C]adenine, [^{14}C]adenosine, [^{14}C]guanine, [^{14}C]guanosine, [^{14}C]hypoxanthine, [^{14}C]inosine, and [^{14}C]xanthine, into nucleotides (Fig. 4). The *Δhgprt* promastigotes exhibited a decreased capacity to metabolize both [^{14}C]hypoxanthine and [^{14}C]guanine, *Δaprt* promastigotes displayed decreased [^{14}C]adenine incorporation, and *Δxprt* parasites were incapable of converting [^{14}C]xanthine to the nucleotide level (Fig. 4). The parental heterozygotes of the three knockout lines converted their substrate nucleobases to anionic metabolites as effectively as wild type promastigotes (data not shown). The *Δhgprt* , *Δaprt* , and *Δxprt* knockouts also robustly took up the three radiolabeled nucleosides, [^{14}C]adenosine, [^{14}C]guanosine, and, [^{14}C]inosine at rates comparable to that of wild type parasites (Fig. 4).

3.4.5. Growth phenotype of *Δhgprt*, *Δaprt*, and *Δxprt* promastigotes

The ability of *Δhgprt, Δaprt, and Δxprt* promastigotes to proliferate in various purine sources was compared (Table 1). Whereas wild type parasites could proliferate in medium supplemented with any of the added purine nucleobases or nucleosides tested, *Δhgprt* promastigotes grew slowly and to a lower maximum density in guanosine, and *Δxprt* promastigotes could not survive

or replicate in medium containing guanine, xanthine, or xanthosine as the sole purine source. All other purine nucleobases and nucleosides could fulfill the purine nutritional requirements of the mutant parasites in the absence of further pharmacological manipulation of the growth medium (Table 1). Addition of 20 μM dCF, however, completely prevented *Δaprt* promastigotes from growing in adenine and decreased their growth in adenosine by ~50%. No effect of dCF on the growth of wild type, *Δhgprt*, and *Δhgprt* promastigotes was detected.

3.4.6. Transformation of wild type, *Δhgprt*, *Δaprt*, and *Δxprt* promastigotes to axenic amastigotes

The ability of *Δhgprt, Δaprt , and Δxprt* promastigotes to transform into axenic amastigotes was then evaluated by transferring the parasites from promastigote to axenic amastigote growth medium (see Materials and Methods). All three knockout lines experienced the same morphological transformation as the wild type LdBob line and were capable of continuous growth at 37 °C and pH 5.5. Western blot analysis revealed that the axenic amastigotes selectively expressed the amastigote-specific A2 markers (15), whereas the promastigote counterparts did not (Fig. 5). Multiple A2 bands were recognized by the A2 monoclonal antibody in axenic amastigote extracts, consistent with the presence of multiple genes encoding A2 proteins of different lengths within the *L. donovani* genome (15, 17, 18).

3.4.7. Expression of HGPRT, APRT, and XPRT in axenic amastigotes

Immunoblotting of wild type LdBob axenic amastigote lysates revealed that axenic amastigotes expressed all three PRT proteins to levels comparable to those found in promastigote extracts (Fig. 6). The size of the protein band recognized by the monospecific polyclonal antisera prepared to each of the proteins corresponded to the size of the polypeptide predicted from the encoded gene (5, 13, 14).

3.4.8. Growth phenotypes of *Δhgprt*, *Δaprt*, and *Δxprt* axenic amastigotes

The ability of wild type and knockout axenic amastigotes to multiply in various purine nucleobases and nucleosides was assessed. The growth phenotypes of the axenic amastigotes of wild type and mutant parasites in naturally occurring purines were essentially identical to their promastigote counterparts (data not shown). In addition, dCF also obliterated the ability of *Δaprt* axenic amastigotes to proliferate with adenine as the sole purine in the culture medium.

3.4.9. [^{14}C]Adenine metabolism in intact wild type and *Δaprt* axenic amastigotes

Previous reports in the literature have indicated that AAH is a promastigote-specific activity not found in *L. donovani* amastigotes (2). Therefore, the abilities of intact wild type and *Δaprt* promastigotes and axenic

amastigotes to metabolize [^{14}C]adenine in the presence and absence of 20 μM dCF were compared (Fig. 7). *Δaprt* promastigotes converted [^{14}C]adenine into nucleotides at approximately half the rate of wild type promastigotes (Fig. 7A). 20 μM dCF abolished the ability of the knockout cells to metabolize [^{14}C]adenine into nucleotides and had virtually no effect on the ability of wild type parasites to metabolize the nucleobase. Similar results were observed with the axenic amastigotes, except that the *Δaprt* cells metabolized [^{14}C]adenine comparably with the wild type parasites (Fig. 7B).

3.4.10. Macrophage infectivity assays

To ascertain whether PRT-deficient null parasites were capable of infecting mammalian cells, parasite infectivity assays were carried out with bone marrow-derived murine macrophages. Wild type, *Δhgprt*, *Δaprt*, and *Δxprt* were all capable of infecting and sustaining infection in the murine macrophages, but the absolute level of infectivity of the three mutants was ~50% of that of the wild type strain (Fig. 8). To ascertain whether this reduction in the overall parasite load could be ascribed specifically to the genetic lesions inserted into the various lines, infectivity studies were also performed on the episomally complemented *Δhgprt*[p*HGPRT*], *Δaprt*[p*APRT*], and *Δxprt*[p*XPRT*] lines. At most, episomal complementation had a modest effect on parasite numbers in the bone-derived macrophage model (Fig. 9). Similar infectivity data with the knockout and

complemented lines were obtained using J774 macrophages as an infection model (data not shown).

3.5. Discussion

In order to test the functional roles that PRTs play in purine salvage in infectivity and amastigote metabolism, *Δhgprt*, *Δaprt*, and *Δxprt* knockouts were created in a virulent *L. donovani* background by targeted gene replacement. Each of these null mutants had been previously constructed in promastigotes of an avirulent, noninfectious strain of *L. donovani* (4, 5), but these strains were only preliminarily characterized and the requirement for PRT involvement in promastigote transformation to the amastigote, amastigote metabolism, infectivity of mammalian host cells, and their ability to proliferate as amastigotes could not be assessed. The LdBob knockouts retained their ability to transform into axenic amastigotes, to infect primary murine macrophages, and to sustain an infection indicating that neither the *Δhgprt*, *Δaprt*, nor *Δxprt* lesions conferred purine auxotrophy upon the amastigotes or obstructed infectivity and amastigote reproduction. Thus, neither HGPRT, APRT, nor XPRT by itself is essential for promastigote or amastigote viability. Furthermore, the ability of *Δhgprt*, *Δaprt*, and *Δxprt* parasites to sustain infection in macrophages, where they reside in the phagolysosome, demonstrates that the mammalian host cell is capable of supplying a multiplicity of different purines to meet the nutritional needs of the pathogen. These purines, presumably nucleosides and/or nucleobases that are transported into the parasite by a battery of equilibrative nucleoside transporters (19), can then be salvaged by PRT enzymes.

The phenotypic characterization of the null mutants revealed some unusual and novel features about the leishmanial purine salvage pathway. The *Δxprt* LdBob line failed to grow in xanthine, xanthosine, and guanine providing genetic proof that XPRT is the major route by which these three purines satisfy the purine nutritional requirement for both promastigotes and axenic amastigotes. Exogenous guanine is presumably transformed to xanthine by guanine deaminase, an activity previously described by Marr and coworkers (1), and a gene encoding a presumptive guanine deaminase activity has been annotated in the *L. major* (LmjF29.0867) and *L. infantum* (LinJ29.1050) genome databases. Although biochemical studies have revealed guanine to be a high affinity substrate for the *L. donovani* HGPRT (14) and *Δxprt* parasites are still capable of taking up [^{14}C]guanine over a short time interval (Fig. 4), it seems likely that the guanine deaminase converts the aminopurine to xanthine, a dead end metabolite for the *Δxprt* mutant, over the 5 day duration of the growth experiment (Table 1). Surprisingly, guanosine can meet the purine nutritional requirement of the *Δxprt* strain. This implies that guanosine, although a substrate for a well characterized nucleoside hydrolase protein that converts guanosine to guanine (20), must have at least one additional but yet uncharacterized metabolic avenue for supplying the parasite purine pool, a pathway that must bypass XPRT.

Previous studies on *L. donovani* extracts have indicated that AAH is expressed exclusively in the promastigote stage of the parasite. AAH is common in bacteria, but its activity has been described in a select very few

eukaryotes, including *Leishmania* (16), *Crithidia* (21), yeast (22), and *Aspergillus* (23). Our studies with the *Δaprt* line and dCF, the AAH inhibitor (16), however, strongly imply that axenic amastigotes do express AAH activity. As observed in Fig. 7, [^{14}C]adenine metabolism in axenic amastigotes, like adenine metabolism in promastigotes, is only abolished by a combination of a genetic lesion in APRT and a pharmacologic block of AAH. These findings indicate that wild type promastigotes and axenic amastigotes have two routes for adenine metabolism, APRT that is abrogated genetically and AAH that is blocked pharmacologically in *Δaprt* parasites treated with dCF.

These genetic studies also impact on theoretical therapeutic paradigms for treating and/or preventing leishmaniasis. The inability of *Leishmania*, as well as other parasites, to synthesize the purine ring *de novo* (1), has bolstered the purine salvage pathway as a therapeutic target for anti-parasitic regimens. Previous studies have demonstrated that the purine salvage pathway of *Leishmania* is myriad and complex (1, 2), but the exact routes by which exogenous or host purines are metabolized by the parasite could not be ascertained by activity measurements in crude lysates (3) or metabolic flux experiments in intact cells (3). Previous genetic studies have confirmed that no single PRT enzyme or AK(3, 4) is essential for the viability or growth of *L. donovani* promastigotes, and these genetic analyses have now proven that neither HGPRT, APRT, nor XPRT by itself is essential for amastigote nutrition and proliferation. Therefore, in order to target the purine salvage process of *L.*

donovani, it is essential to inhibit a multiplicity of salvage enzymes. The availability of *Δhgprt/Δaprt/ak*$^-$ (10) and *Δxprt/Δaprt/ak*$^-$ (unpublished) strains strongly suggest that *L. donovani* can survive and proliferate with only a functional XPRT or HGPRT, respectively, and that all exogenous purines can be funneled to either hypoxanthine or xanthine, a conjecture that is supported by the results of this paper. Thus, pharmacological inhibition of both HGPRT and XPRT can be proposed as the sole rational therapeutic paradigm that targets the unique but intricate purine salvage pathway of this protozoan pathogen with inhibitors, but this model will need to be experimentally verified using genetic approaches.

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3.6 Figures

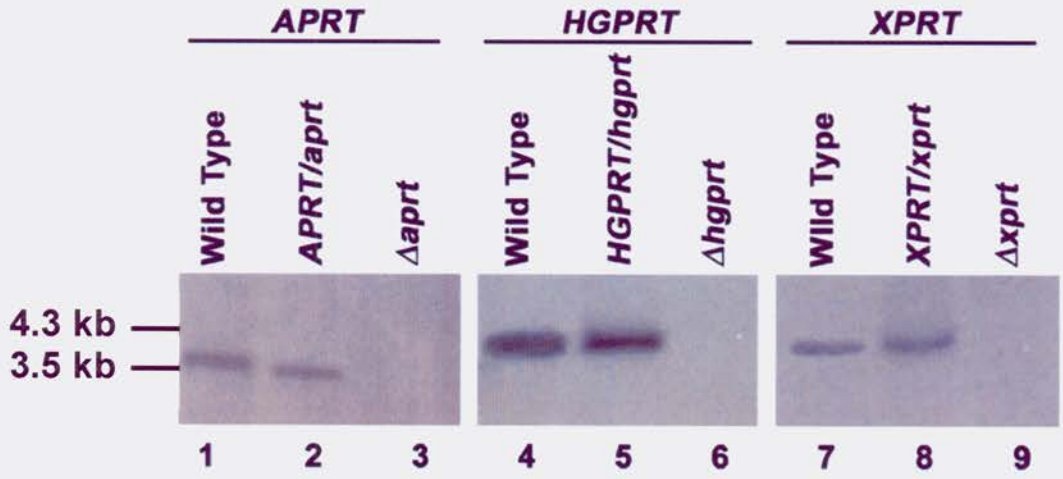

Figure 1. Southern blot analysis of PRT knockouts. 25 μg of total genomic DNA from wild type, heterozygous and knockout parasites was digested with either BamHI/SalI (lanes 1-3) or EcoRI (Lanes 4-9), fractionated on a 0.8% agarose gel, and blotted onto nylon membranes. Blots were hybridized under high stringency conditions with probes to the *APRT* (lanes 1-3), *HGPRT* (lanes 4-6) or *XPRT* (lanes 7-9) coding regions.

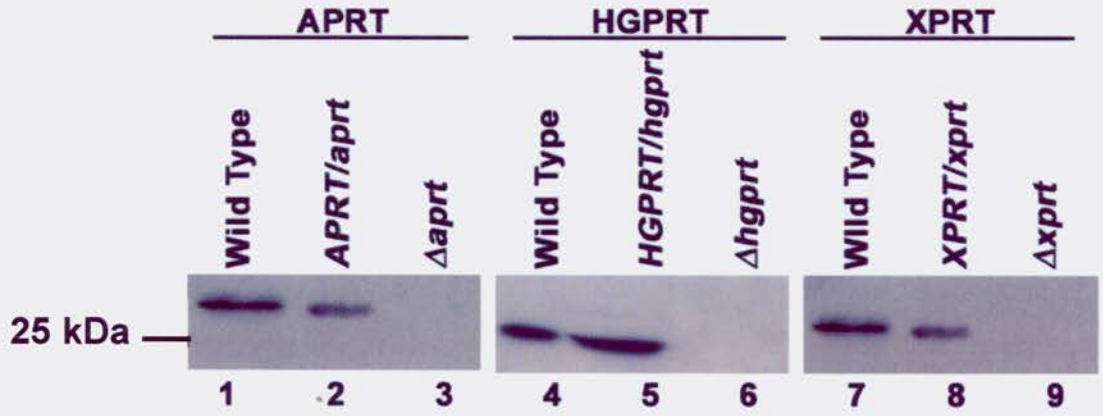

Figure 2. Immunoblot analysis of PRT expression in wild type, heterozygous, and homozygous mutants. Lysates of exponentially growing wild type, heterozygous and null mutants were analyzed by western blotting using polyclonal antisera to APRT(lanes 1-3), HGPRT (lanes 4-6), or XPRT (lanes 7-9).

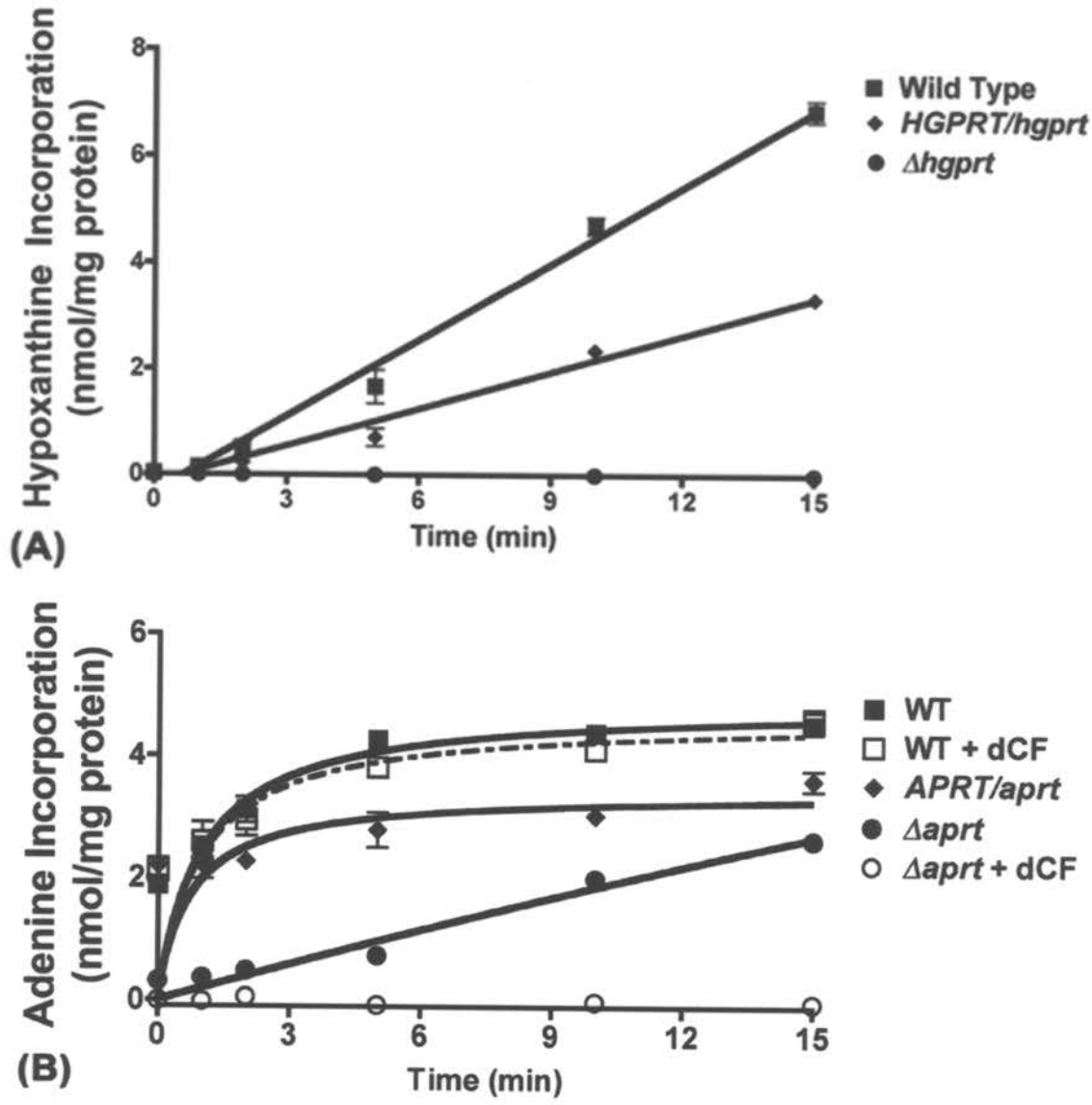

Figure 3. Hypoxanthine and adenine incorporation in cell extracts. The ability of extracts prepared from wild type, *HGPRT/hgprt* and *Δhgprt* cells to incorporate [^{14}C]hypoxanthine and PRPP into nucleotides was measured as described in "Materials and Methods" (Panel A). The amount of incorporation of [^{14}C]adenine and PRPP into nucleotides by extracts prepared from wild type, *APRT/aprt* and *Δaprt* cells was quantified (Panel B).

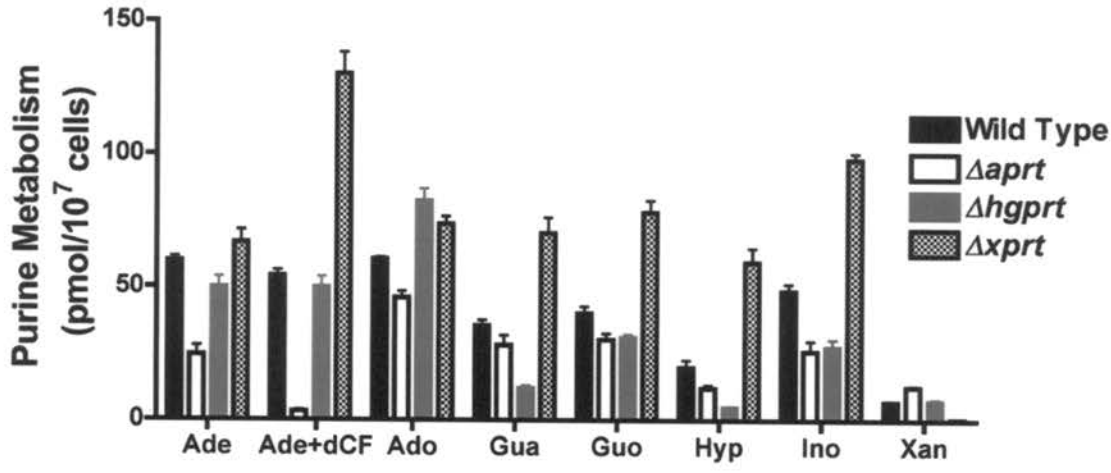

Figure 4. Purine incorporation into intact *L. donovani* promastigotes. The ability of wild type, *Δaprt*, *Δhgprt* and *Δxprt* promastigotes to incorporate various purine sources into nucleotides was measured over a two h time course.

Purine	Cell Line			
	Wild Type	*Δaprt*	*Δhgprt*	*Δxprt*
Adenine	++++	++++	++++	++++
Adenine+dCF	++++	—	++++	++++
Adenosine	++++	++++	++++	++++
Adenosine+dCF	++++	++	++++	++++
Guanine	++++	+++	+++	—
Guanosine	++++	+++	++	+++
Hypoxanthine	++++	++++	++++	++++
Inosine	++++	++++	+++	++++
Xanthine	++++	++++	+++	—
Xanthosine	++++	++++	+++	—

Table 1. Growth of promastigotes in various purine sources The number of (+) symbols represents the level of growth relative to all cell lines, (—) indicates no growth in the specified condition.

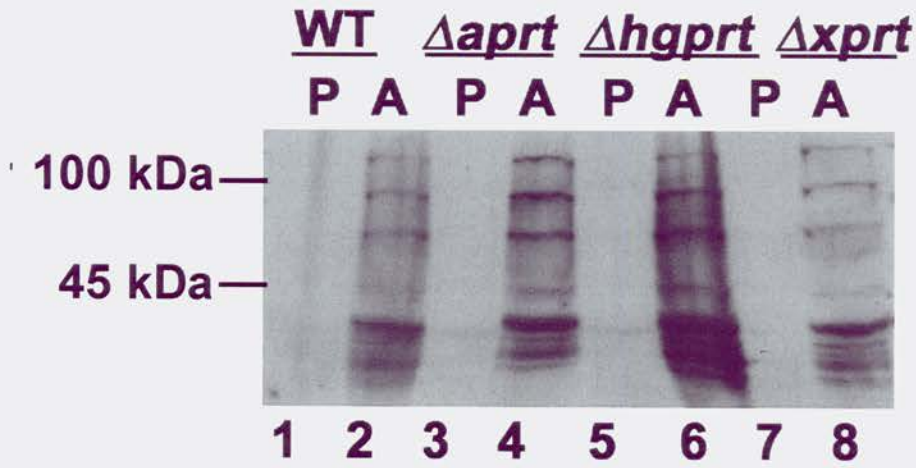

Figure 5. Expression of A2 stage-specific markers in axenic amastigotes. Lysates from wild type (lanes 1, 2), *Δaprt* (lanes 3, 4), *Δhgprt* (lanes 5, 6), and *Δxprt* (lanes 7, 8) promastigotes (P) (lanes 1, 3, 5, 7) and axenic amastigotes (A) (lanes 2, 4, 6, 8) were analyzed by western blotting using mouse monoclonal antibodies to the amastigote-specific A2 protein family [15].

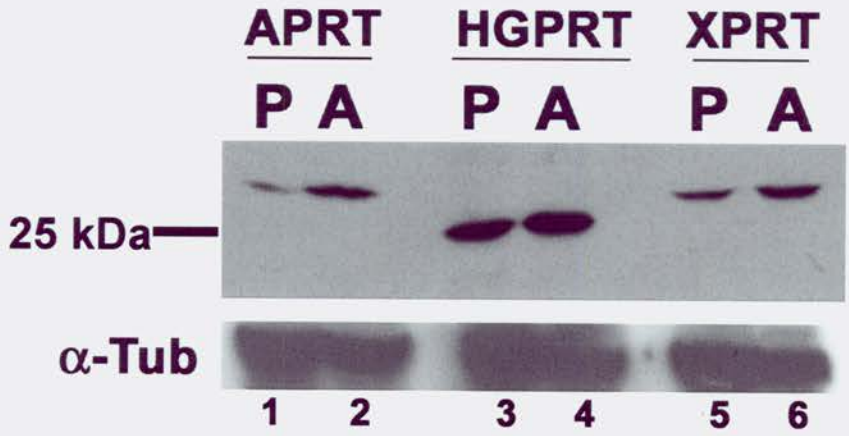

Figure 6. Expression of PRT proteins in promastigotes and amastigotes. Immunoblot analysis was performed with antisera specific for *L. donovani* APRT, HGPRT and XPRT on lysates from promastigotes (P) (lanes 1, 3, 5) and axenic amastigotes (A) (lanes 2, 4, 6). The amount of cell lysate loaded onto each lane of the gel was normalized with α-tubulin antisera.

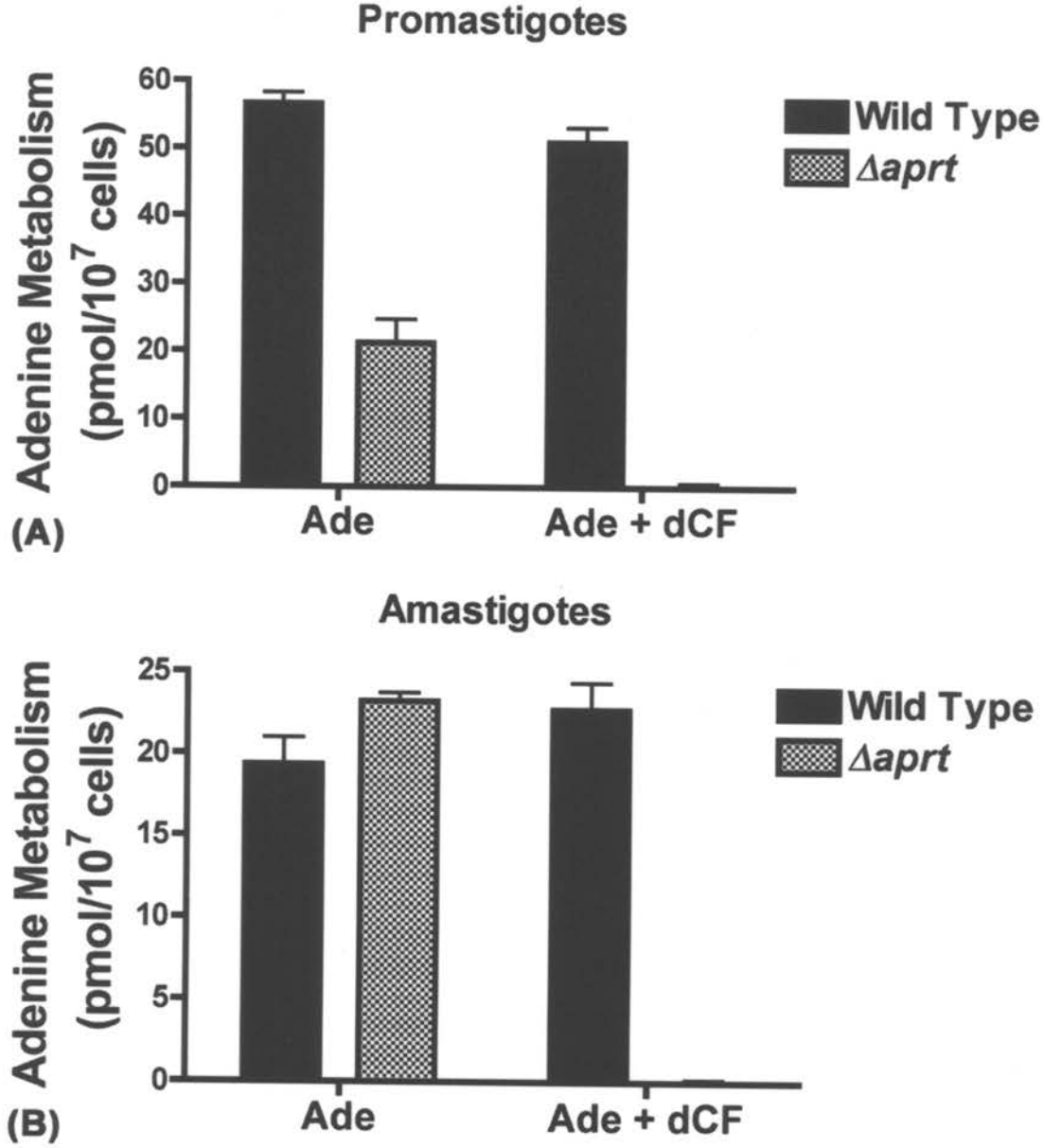

Figure 7. Adenine metabolism in intact promastigotes and amastigotes. The capacity of live promastigotes to incorporate [^{14}C]adenine into phosphorylated products was measured over the duration of 15 min in the presence and absence of 20 μM dCF (Panel A). The ability of intact amastigotes

to incorporate [^{14}C]adenine into phosphorylated products was measured under the same conditions (Panel B).

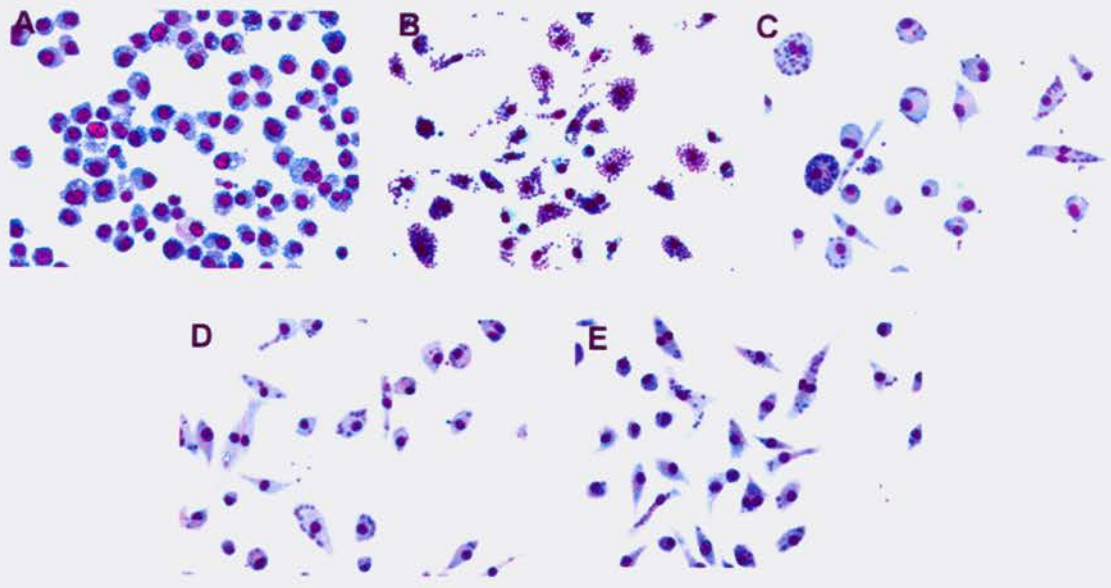

Figure 8. Bone marrow-derived macrophages infected with *L. donovani* parasites. Infections of bone marrow-derived macrophages were accomplished as described in "Materials and Methods". Uninfected macrophages (Panel A) and macrophages infected with wild type (Panel B), *Δaprt* (Panel C), *Δhgprt* (Panel D), and *Δxprt* (Panel E) parasites are depicted.

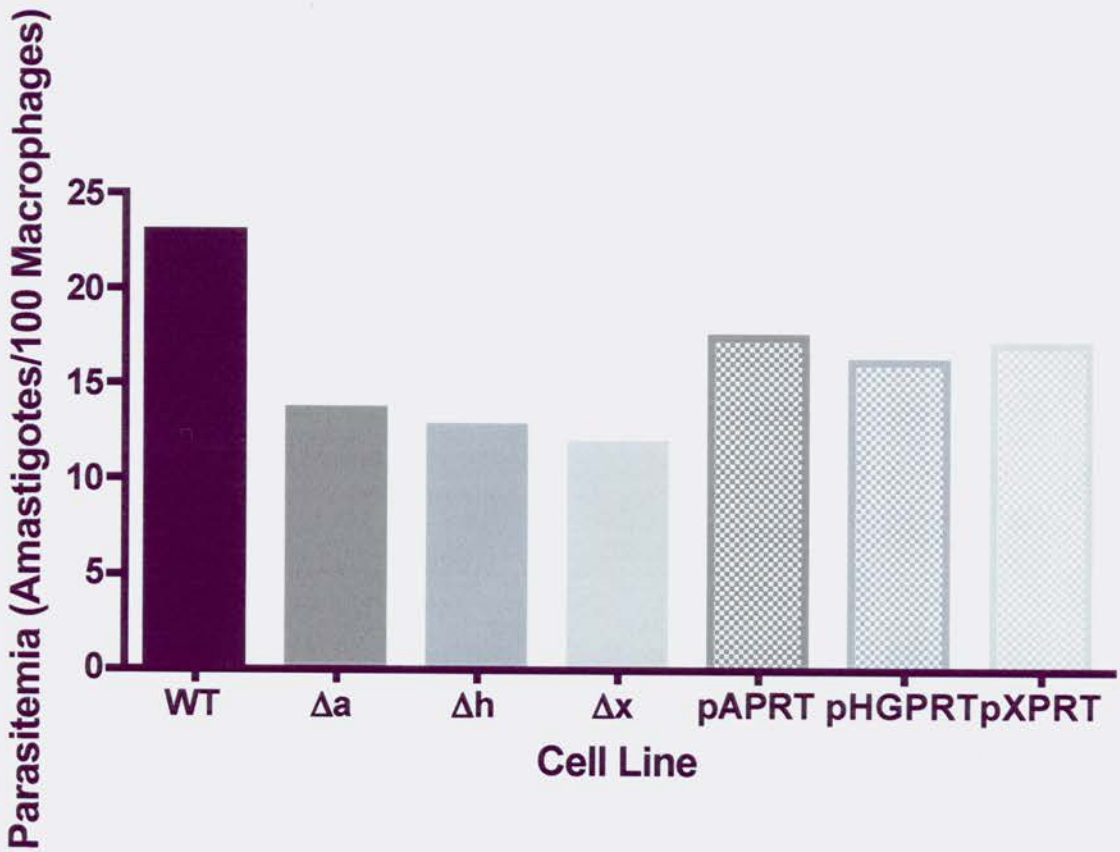

Figure 9. Parasitemia of wild type, mutant and episomally complemented cell lines. The number of wild type (WT), *Δaprt* (Δa), *Δhgprt* (Δh), *Δxprt* (Δx), *Δaprt*[p*APRT*] (pAPRT), *Δhgprt*[p*HGPRT*] (pHGPRT), and *Δxprt*[p*XPRT*] (pXPRT) parasites in bone marrow-derived macrophages was enumerated visually after staining with Diff-Quick. One hundred macrophages from each infection were counted.

3.7. References

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Chapter 3 Appendix

Supplementary Information for the Genetic Analysis of *Leishmania donovani* Mutants Deficient in Purine Phosphoribosyltransferases

3.A.1. Introduction

Detailed in this appendix are several experiments and data figures that were either summarized or omitted from Chapter 3 for publication purposes. Several of these experiments confirm the existence of an active adenine aminohydrolase (AAH) in amastigotes, an activity previously thought not to be present. Experimental procedures (Materials and Methods), results and a brief discussion are included here.

3.A.2. Materials and methods

3.A.2.1. Antibody production and purification

The LdAAH sequence was identified within the *L. major* sequence database (http://www.genedb.org/genedb/leish/) using *S. cerevisiae* aah1, as a blast query. The *L. major aah* gene was PCR-amplified from genomic DNA and used as a probe to isolate the gene from an *L. donovani* cosmid library using standard techniques (1). The sequence of the *LdAAH* gene has been published (2), and a full genetic and biochemical analysis of *Ldaah* and LdAAH will be described in a future publication. The AAH protein sequence was sent to Bio-synthesis Inc. (Lewisville, TX) and computer algorithms were used to predict a peptide sequence from the protein that would be the most antigenic and hydrophilic. The sequence KDLREKRIPLT (amino acids 248-259 of the protein) was chosen and used to generate polyclonal anti-sera in rabbits. The antibody was purified against the peptide according to the instructions provided with the Amino-Link Coupling Gel and Immobilization kit (Pierce Biotechnology Inc. Rockford, IL), and an ELISA was used to test the purified antibody for its specificity against LdAAH protein that had been expressed in the pET 200D/-TOPO ® vector (Invitrogen, Carlsbad, CA) in BL21 Star™ cells and purified on Ni-NTA agarose (Invitrogen, Carlsbad, CA).

3.A.2.2. Thin layer chromatography

$6X10^7$ parasites were spun down, washed two times in PBS and resuspended in 30 µl of either promastigote- or amastigote-specific medium lacking FBS, BSA and hemin. To initiate the reaction 67 µM adenine was added to the resuspended parasites. At each time point 5 µl ($1X10^7$) of cells was transferred into an eppendorf tube containing 5 µl of concentrated acetic acid and pipeted up and down to lyse the cells and stop the reaction. The cell/acid mixture was centrifuged at 13,000 rpm for 30 seconds and the supernatant spotted on a polyethyleneimine (PEI) cellulose plastic-backed TLC plate (Scientific Adsorbants Inc., Atlanta, GA). The plates were chromatographed as in (3) and set to expose film for 2 days at -80 ºC.

3.A.2.3. Immunofluorescence analysis

Immunofluorescence analysis (IFA) was performed as in (4) except that cells were permeablized with 0.02%, rather than 0.1%, Triton X-100 in order to avoid destruction of the macrophages. Primary antibodies were used at a dilution of 1:100 and secondary at 1:5,000. The mounting medium contained 0.75 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Parasites were detected on a Zeiss Axiovert 200M scope (Carl Zeiss Microimaging, Thornwood, NY) using a 60X oil immersion lens. Photographs were taken with an AxioCam MRm camera (Carl Zeiss Microimaging).

3.A.3. Results

3.A.3.1. Enzyme assays in promastigote extracts

Incorporation of [^{14}C]adenosine was measured in extracts of wild type and *Δaprt* cells. [^{14}C]adenosine incorporation was similar in both cell lines and was reduced only slightly by the addition of 20 μM dCF (Fig. 1).

3.A.3.2. Purine metabolism in intact promastigotes

The ability of intact wild type and knockout parasites to metabolize exogenous radioactive purine nucleobases into phosphorylated products was gauged. The data for wild type and knockout parasites was summarized in Chapter 3, Fig. 4, but the complete time course, including data for the heterozygotes, is shown here. Wild type parasites were capable of transforming [^{14}C]hypoxanthine, [^{14}C]adenine, and [^{14}C]xanthine into nucleotides, while the *HGPRT/hgprt*, *APRT/aprt*, and *XPRT/xprt* heterozygote promastigotes could incorporate [^{14}C]hypoxanthine, [^{14}C]adenine, and [^{14}C]xanthine, respectively, nearly as efficiently as the wild type parental strain (Fig. 2A-C). The *Δhgprt* and *Δaprt* promastigotes also metabolized [^{14}C]hypoxanthine and [^{14}C]adenine, respectively, nearly as effectively as heterozygous parasites, whereas no [^{14}C]xanthine metabolism into nucleotides was detected in *Δxprt* parasites (Fig. 2A-C). The hypoxanthine metabolism seen in *Δhgprt* cells (Fig. 2A) is a result of XPRT utilization of hypoxanthine, an activity not detected in the experiments with cell lysates (section 3.3.4) because we cannot detect XPRT activity in parasite

extracts for technical reasons. The addition of dCF also dramatically reduced adenine metabolism in *Δaprt* promastigotes (Fig. 2B). Additionally, metabolism of [^{14}C]adenosine was measured in wild type and *Δaprt* cells. Both cell lines incorporated adenosine into phosphorylated products at the same level, and this incorporation was not dramatically affected by the addition of 20 μM dCF in either cell line (Fig. 2D).

3.A.3.3. Growth phenotypes of *Δhgprt*, *Δaprt*, and *Δxprt* axenic amastigotes

The ability of wild type and knockout axenic amastigotes to multiply in various purine nucleobases and nucleosides was assessed (Table 1). The growth phenotypes of the axenic amastigotes of wild type and mutant parasites were the same as their promastigote counterparts. Wild type and *Δhgprt* axenic amastigotes could grow on all purines tested, while *Δxprt* parasites could not grow on guanine, xanthine, or xanthosine.

3.A.3.4. Enzyme assays with lysates from axenic amastigotes

PRT assays were carried out with cell-free extracts of wild type, *Δhgprt*, *Δaprt*, and *Δxprt* axenic amastigotes. These data mirrored the purine metabolism profile of promastigotes except that no adenosine metabolism was detected in any of the cell lines, and no AAH activity was measured in the *Δaprt* mutant (Fig. 3A). It was possible that the latter two discrepancies were due to an inactivation the AAH activity during the cell lysate preparation. The full 15 minute time course for wild type and *Δaprt* cell extracts is shown in Fig. 3B. From this

data it appears that no [^{14}C]adenine is incorporated by extracts of *Δaprt* axenic amastigotes in the absence or presence of 20 μM dCF.

3.A.3.5. Adenine and adenosine metabolism in live amastigotes

In order to assess the presence of AAH in axenic amastigotes, the ability of intact wild type and *Δaprt* amastigotes to incorporate [^{14}C]adenine and [^{14}C]adenosine was measured (Fig. 4). The addition of 20 μM dCF had a minimal effect on the ability of wild type cells to incorporate adenine, whereas it eliminated this capacity in *Δaprt* cells (Fig. 4A). The ability to detect adenine metabolism in *Δaprt* amastigotes, and the finding that this activity can be inhibited by dCF, indicates the presence of an active AAH enzyme in the intracellular form of *Leishmania donovani* (Fig. 4A). Adenosine metabolism was detected in both cell lines and was not inhibited by the addition of 20 μM dCF in either wild type or *Δaprt* amastigotes (Fig. 4B).

3.A.3.6. Thin layer chromatography

As an additional effort to confirm the presence of AAH activity in amastigotes, TLC was carried out using [^{14}C]adenine as a substrate for wild type and *Δaprt* amastigotes. Wild type promastigotes were used as a control in these experiments (Fig. 5). In all three cell lines the majority of the radiolabelled adenine substrate was converted to hypoxanthine product within an hour (Fig. 5A). The level of adenine conversion to hypoxanthine in amastigotes was slightly lower than that of the promastigotes. The experiment was repeated in the

presence of 20 μM dCF, the addition of which obliterated the metabolism of adenine in all three cell forms (Fig. 5B). These data further confirm that amastigotes express an AAH activity.

3.A.3.7. Western blot detection of LdAAH in both promastigotes and amastigotes

Western blotting was performed as described in the "Materials and Methods" section of Chapter 3 (Section 3.3.6). A western blot analysis of wild type promastigote and amastigote cell-free extracts further confirmed the presence of AAH protein in both forms of the parasite (Fig. 6).

3.A.3.8. Immunofluorescence analysis

In order to visualize whether AAH was expressed in intracellular amastigotes, purified antibody raised against an AAH peptide was used against macrophages that had been infected with wild type LdBob parasites. The parasites had been allowed to infect for 36 hours to ensure their transformation into amastigotes. Oregon green-conjugated secondary antibody revealed the presence of AAH within intracellular amastigotes (Fig. 7A). The cells were counterstained with DAPI to allow visualization of parasite and macrophage DNA (Fig. 7B). Fluorescently stained amastigotes can be seen inside macrophages, (merged Fig. 7C) a result that further demonstrates the expression of AAH in amastigotes. As a control, and to begin investigating the subcellular location of AAH, IFA was also performed on promastigotes (Fig. 7D). This experiment

revealed a possible punctate pattern of staining for AAH in promastigotes. Unfortunately, IFA images of axenic amastigotes were not able to be obtained at the same time as those of promastigotes.

3.A.4. Discussion

Previous genetic studies have confirmed that no single PRT enzyme or AK is essential for the viability or growth of *L. donovani* promastigotes, and recent genetic analyses have now proven that neither HGPRT, APRT, nor XPRT is essential for amastigote nutrition and proliferation. Growth studies revealed similarities in the purine salvage pathways of promastigotes and amastigotes regarding the metabolism of 6-aminopurines. Whereas wild type, *Δhgprt,* and *Δxprt* parasites can all grow on adenine and adenosine in the presence of dCF, promastigotes and amastigotes of the *Δaprt* knockout can only grow on these two purines in the absence of dCF, an inhibitor of AAH activity whose presence in the culture medium prevents adenine conversion to hypoxanthine.

These data demonstrate that wild type promastigotes and amastigotes have two routes for adenine metabolism, APRT that is abrogated genetically and AAH that is blocked pharmacologically in *Δaprt* parasites treated with dCF. The demonstration of AAH activity and visualization of LdAAH in intact amastigotes contradicts an experiment done by Looker, Berens and Marr in 1983 in which they measured AAH activity in amastigote extracts. An experiment described in this section in which there was no incorporation of [^{14}C]adenine into nucleotides by *Δaprt* amastigote extracts agrees with the data of Looker, Berens and Marr (5) (Fig. 3B), but disagrees with the data produced from purine metabolism studies in live cells which are also described in this here (Fig. 4). The discrepancy between the experimental outcome in live cells versus cellular extracts may have a simple explanation: The aforementioned researchers and I prepared our

amastigote extracts by sonication, and it is possible that we may have destroyed the AAH activity during extract preparation. There is very little manipulation of the cells when assaying for activity in intact parasites, therefore the potential for inactivating any metabolic activities is eliminated. Furthermore, I was also able to determine robust adenosine metabolism in intact wild type and *Δaprt* amastigotes, whereas very little adenosine incorporation was detected in any of the amastigote extracts from wild type or the three single PRTase mutants (Fig. 3A), thus giving additional weight to the idea that some metabolic activities may be disrupted during extract preparation.

It could be argued that the axenic amastigotes aren't true amastigotes and that the AAH activity detected was due to residual promastigote characteristics that might have remained after transformation into axenic amastigotes. To address this question, an immunofluorescence assay was carried out using monospecific AAH antibodies that had been purified against the AAH peptide that was used to generate the AAH antibody (Fig. 7). AAH protein can be visualized in intracellular amastigotes after a 2-day infection, indicating that this protein is present in amastigotes. Finally, the punctate staining seen in promastigotes indicates that AAH is located in the glycosome. The amino acid sequence of LdAAH does not appear to contain a PTS-1, but has a putative PTS-2 (6-8). Other experiments, including colocalization with other known glycosomal proteins, are necessary in order to definitively confirm the location of AAH. In addition, a full genetic and biochemical analysis of AAH has been initiated and will be carried out in the future.

3.A.5. Figures

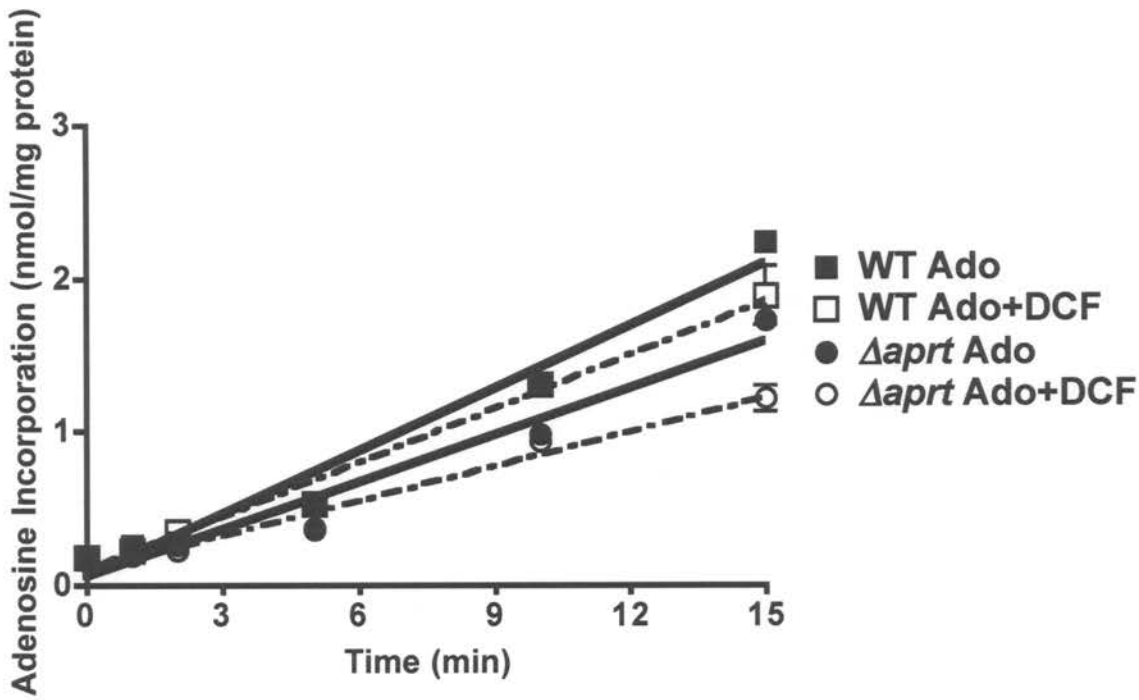

Figure 1. Adenosine incorporation in cell extracts. The ability of extracts prepared from wild type and *Δaprt* cells to incorporate [^{14}C]adenosine and PRPP into phosphorylated nucleosides was measured.

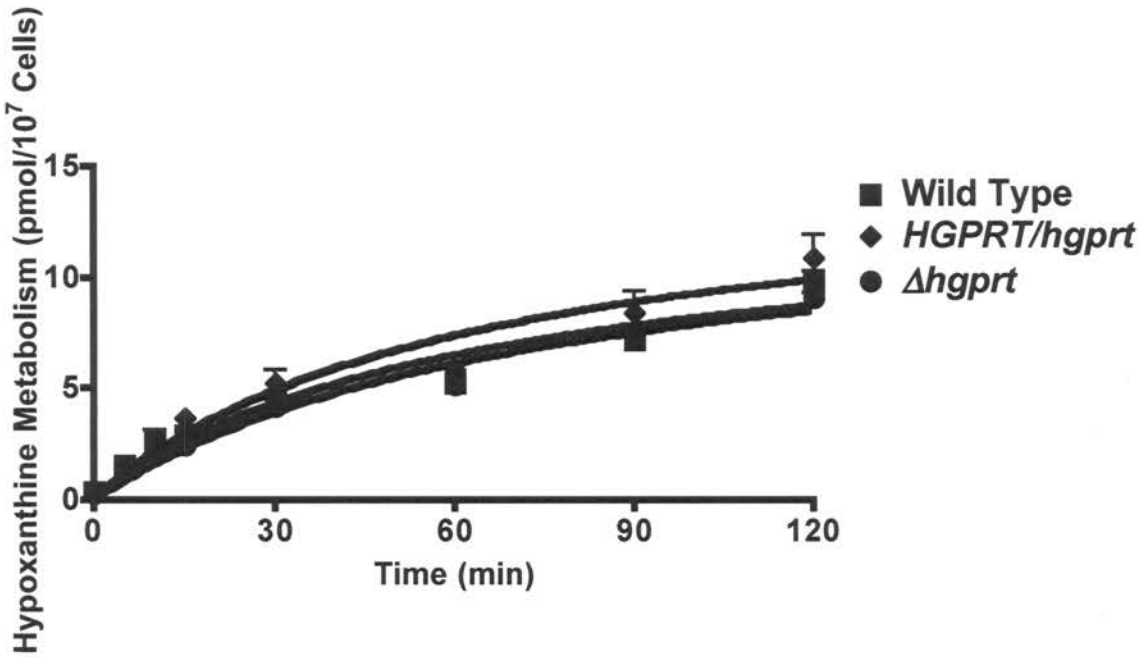

Figure 2A. Hypoxanthine metabolism in intact parasites. The capacity of live parasites to convert [^{14}C]hypoxanthine into phosphorylated products was measured in wild type, *HGPRT/hgprt* and *Δhgprt* cells that had been spun down, washed and resuspended in medium that lacked purine, serum and hemin. [^{14}C]hypoxanthine metabolism was measured over a 2 hour time course.

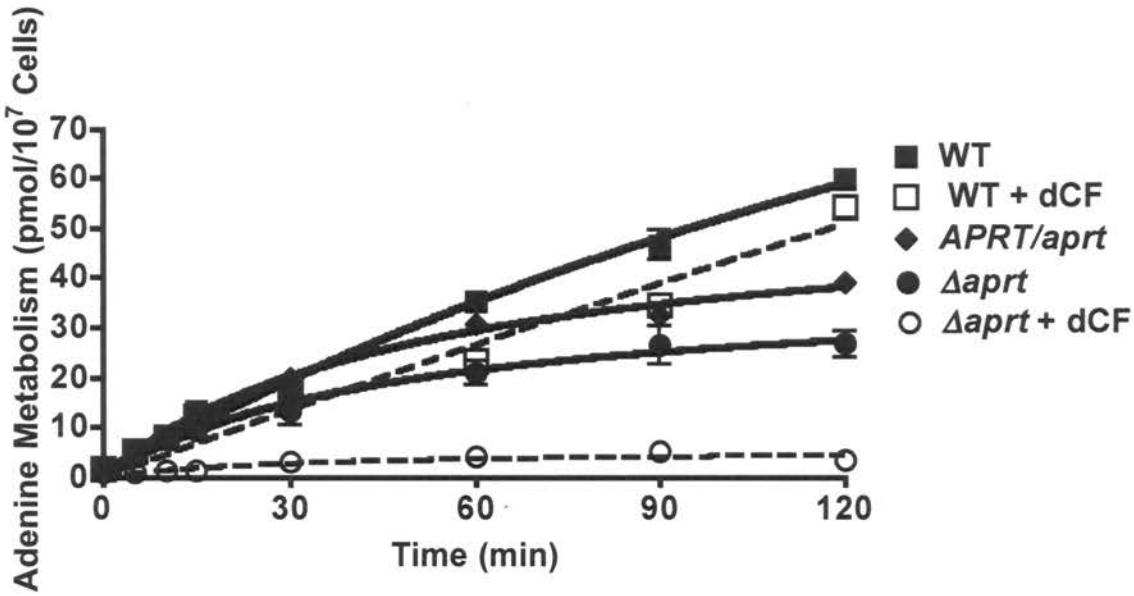

Figure 2B. Adenine metabolism in intact parasites. [^{14}C]adenine metabolism was measured in wild type, *APRT/aprt* and *Δaprt* cells in the presence and absence of 20 μM dCF.

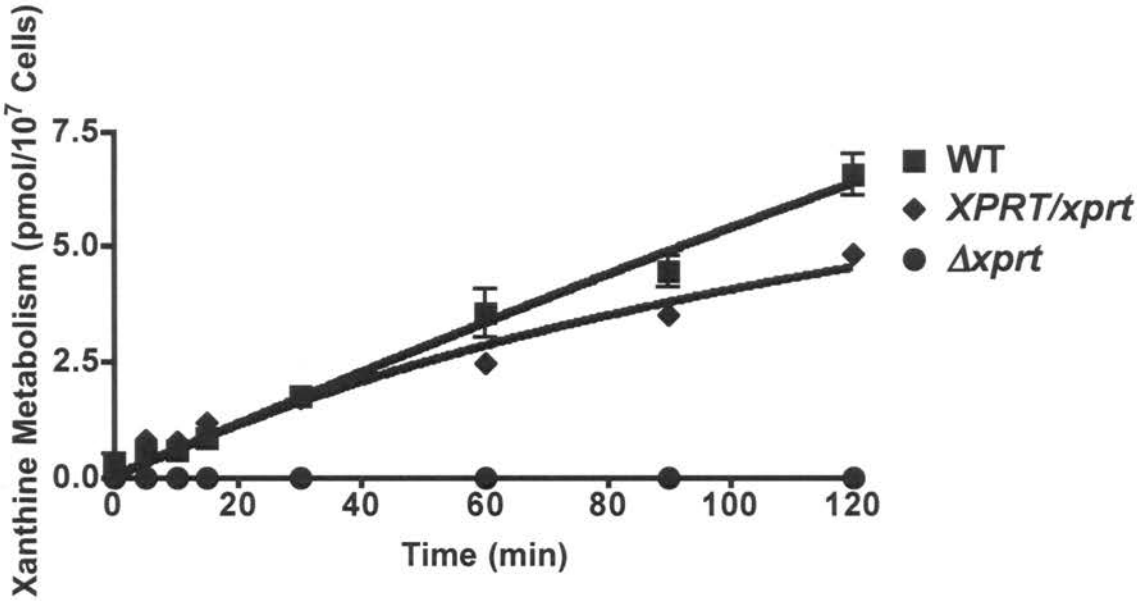

Figure 2C. Xanthine metabolism in intact parasites. The ability of wild type, *XPRT/xprt* and *Δxprt* parasites to metabolize [^{14}C]xanthine was measured over a 2 hour time course.

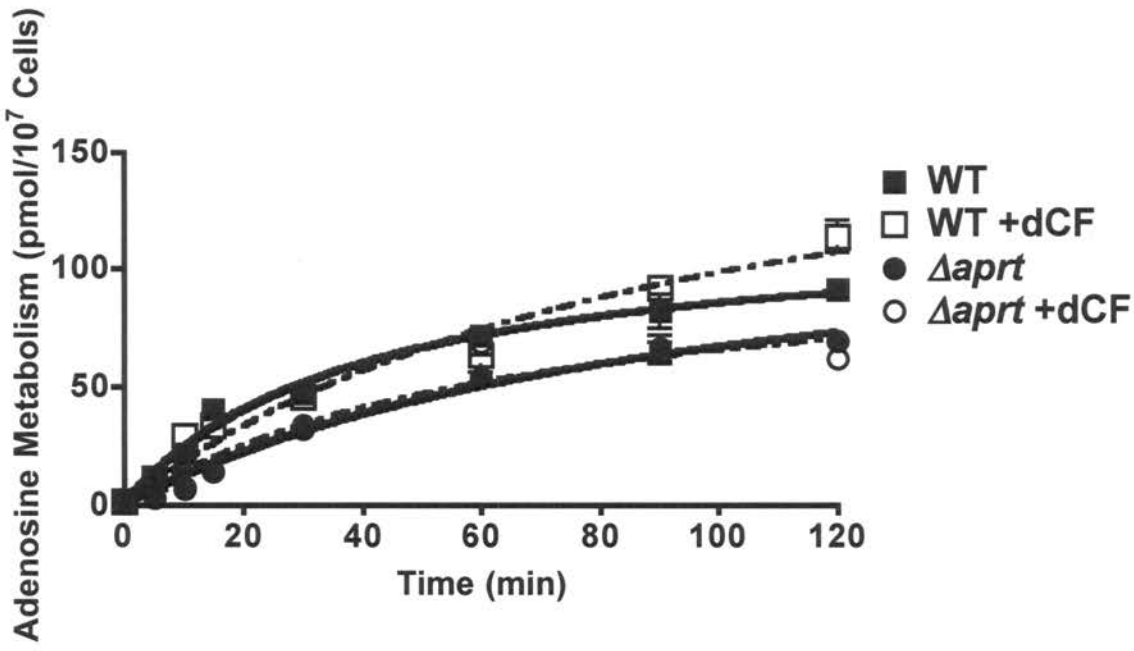

Figure 2D. Adenosine metabolism in intact parasites. [^{14}C]adenosine metabolism in intact wild type, and *Δaprt* parasites was measured in the presence and absence of 20 μM dCF.

Purine	Cell Line			
	Wild Type	*Δaprt*	*Δhgprt*	*Δxprt*
Adenine	++++	++++	++++	++++
Adenine+dCF	++++	—	++++	++++
Adenosine	++++	++++	++++	++++
Adenosine+dCF	++++	++	++++	++++
Guanine	++++	++++	+++	—
Guanosine	++++	++++	++	++
Hypoxanthine	++++	++++	++++	++++
Inosine	++++	++++	+++	++++
Xanthine	++++	++++	+++	—
Xanthosine	++++	++++	+++	—

Table 1. Growth of axenic amastigotes in different purine sources. The number of (+) symbols represents the level of growth relative to all cell lines. (—) indicates no growth in the specified condition

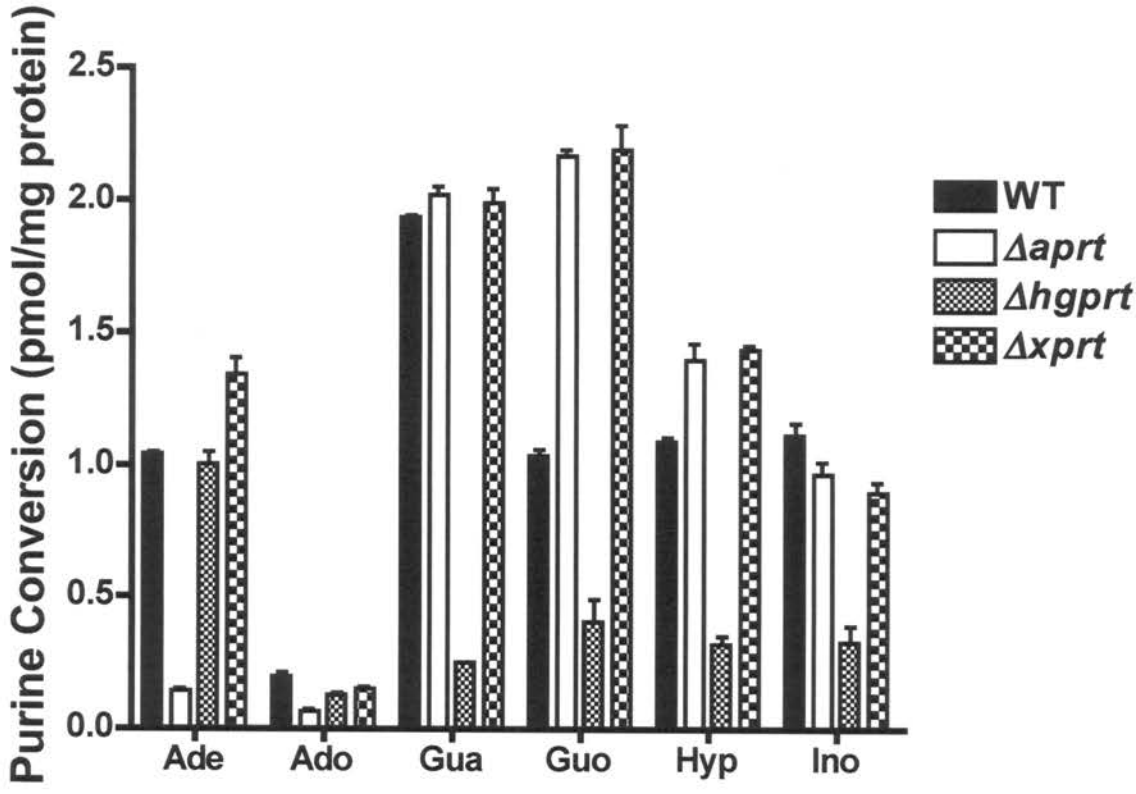

Figure 3A. Purine incorporation in amastigote cell extracts. The incorporation of a variety of [^{14}C] purine sources was assayed in extracts prepared from wild type, *Δaprt*, *Δhgprt* and *Δxprt* axenic amastigotes.

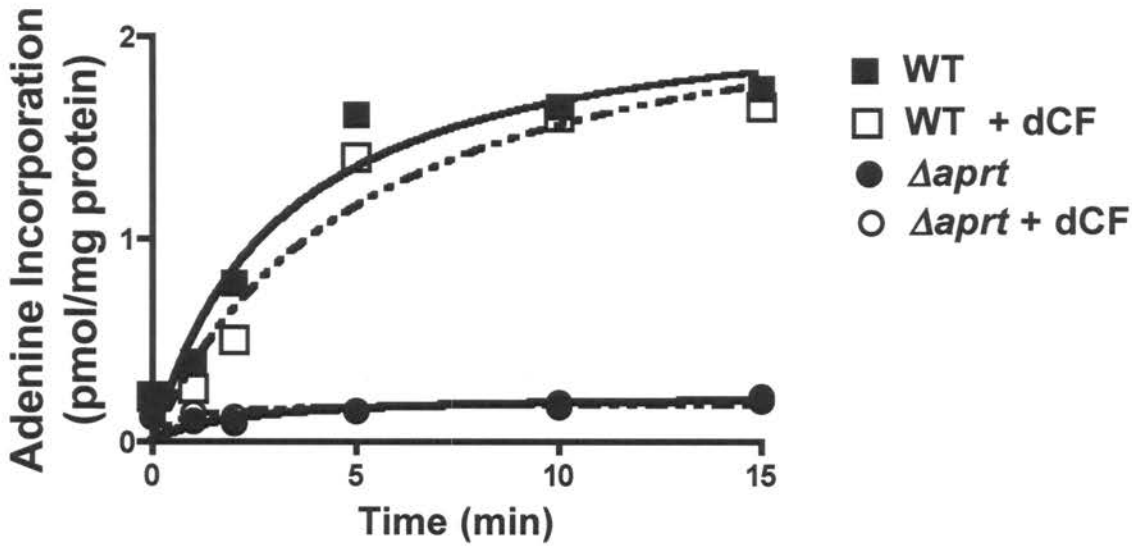

Figure 3B. [^{14}C] adenine conversion in amastigote extracts. The ability of cell extracts prepared from wild type and *Δaprt* amastigotes to incorporate [^{14}C] adenine was measured over a 15 minute time course in the presence and absence of 20 μM dCF.

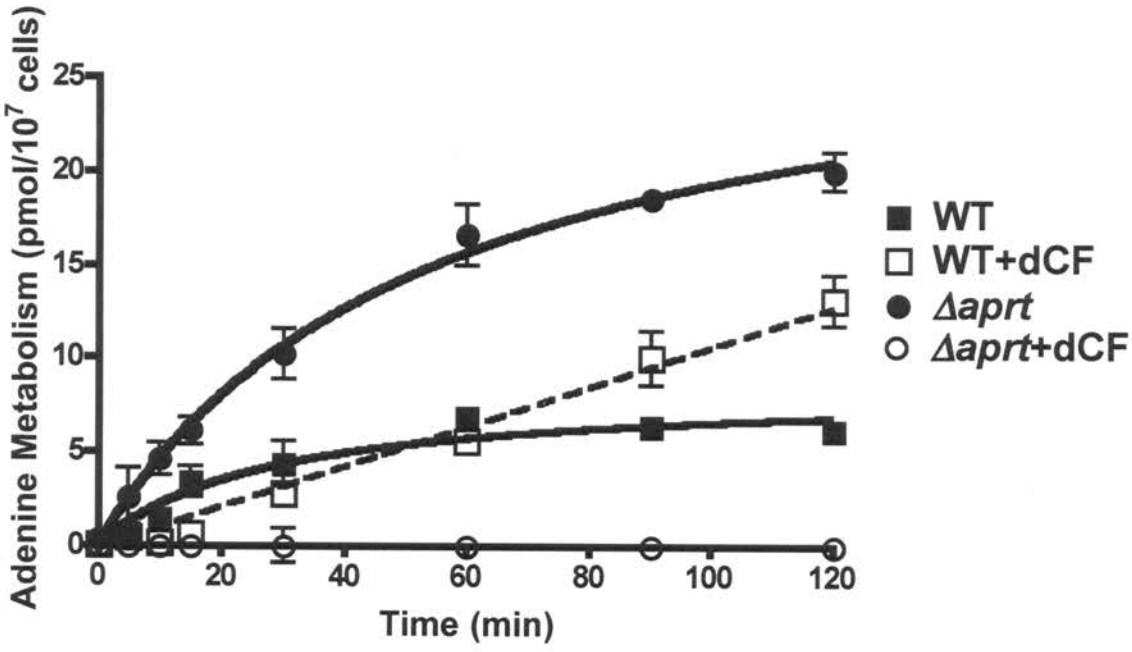

Figure 4A. Adenine metabolism in intact amastigotes. The metabolism of [^{14}C]adenine in wild type and *Δaprt* axenic amastigotes was quantified in live parasites that had been spun down, washed and resuspended in amastigote media lacking any exogenous purine in the presence and absence of 20 μM dCF.

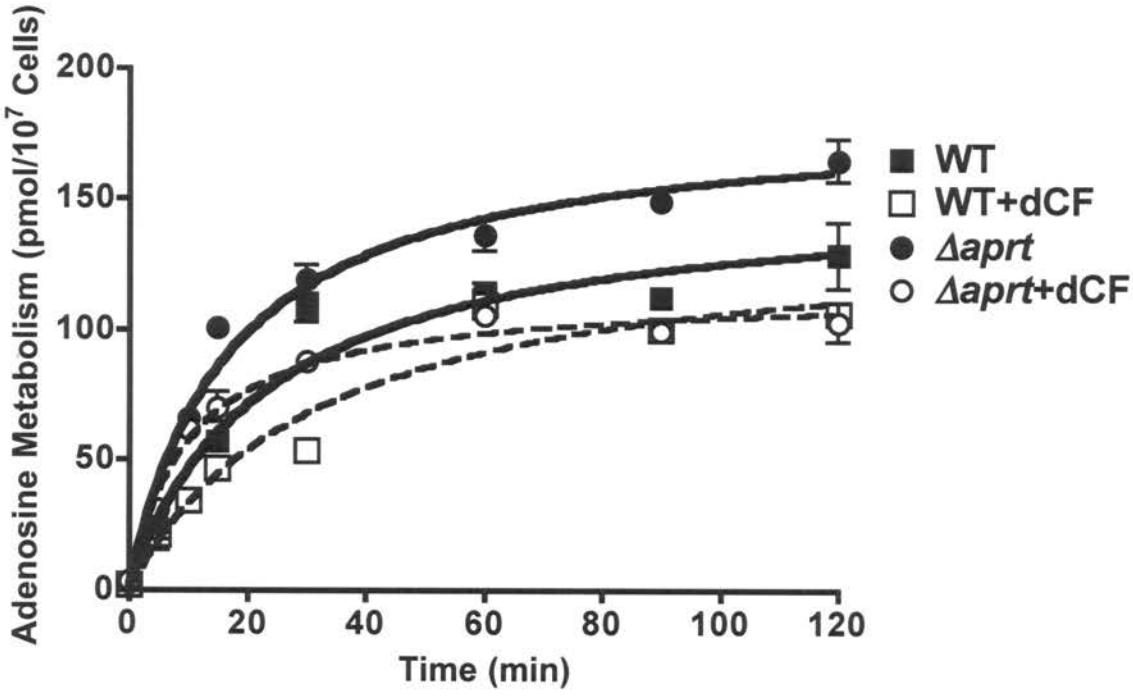

Figure 4B. Adenosine metabolism in intact amastigotes. The ability of wild type and *Δaprt* axenic amastigotes to metabolize [^{14}C]adenosine was measured in intact parasites in the absence and presence of 20 μM dCF

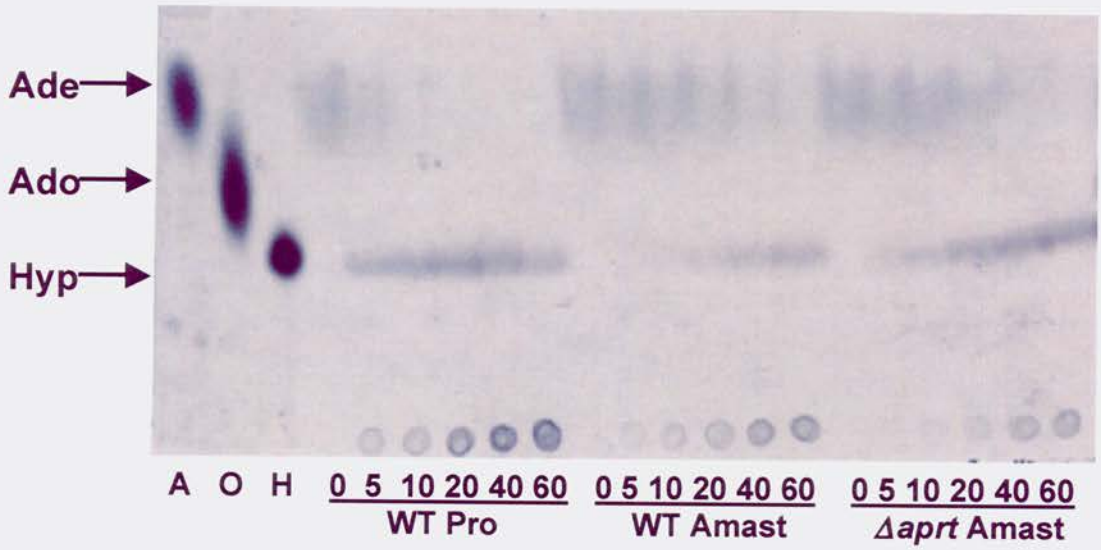

Figure 5A. Thin layer chromatography in the absence of dCF. TLC was performed as described in "Materials and Methods" (A) indicates adenine, (O) adenosine and (H) hypoxanthine, numbers are the time-points in minutes.

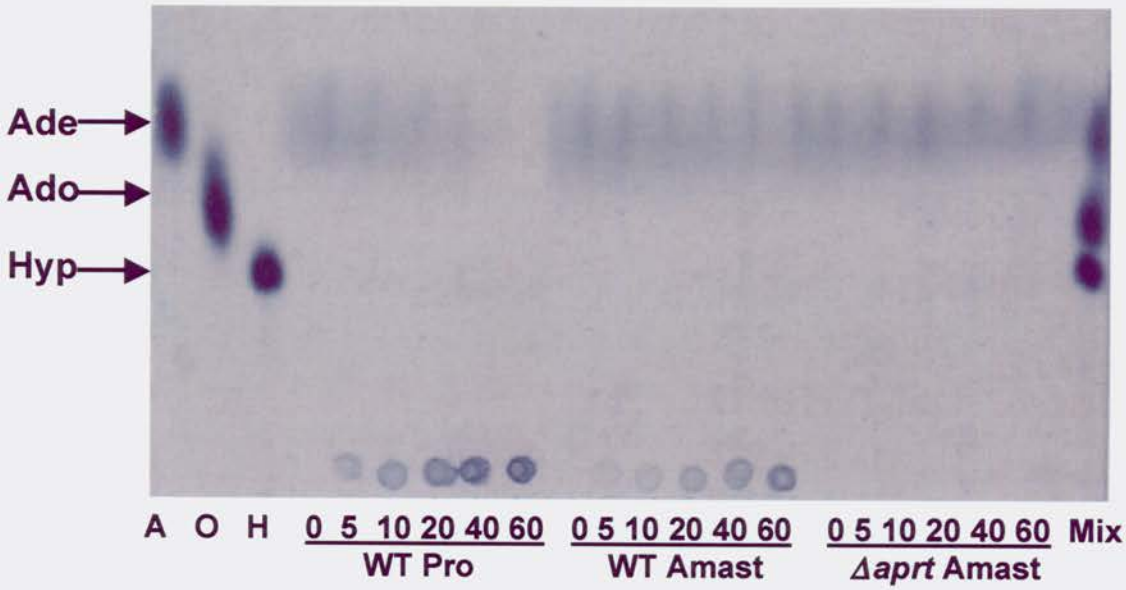

Figure 5B. Thin layer chromatography in the presence of dCF. The TLC was repeated in the presence of 20 μM dCF and [^{14}C]adenine. (A) indicates adenine, (O) adenosine and (H) hypoxanthine, numbers are the time-points in minutes. "Mix" indicates a separated mixture of adenine, adenosine and hypoxanthine.

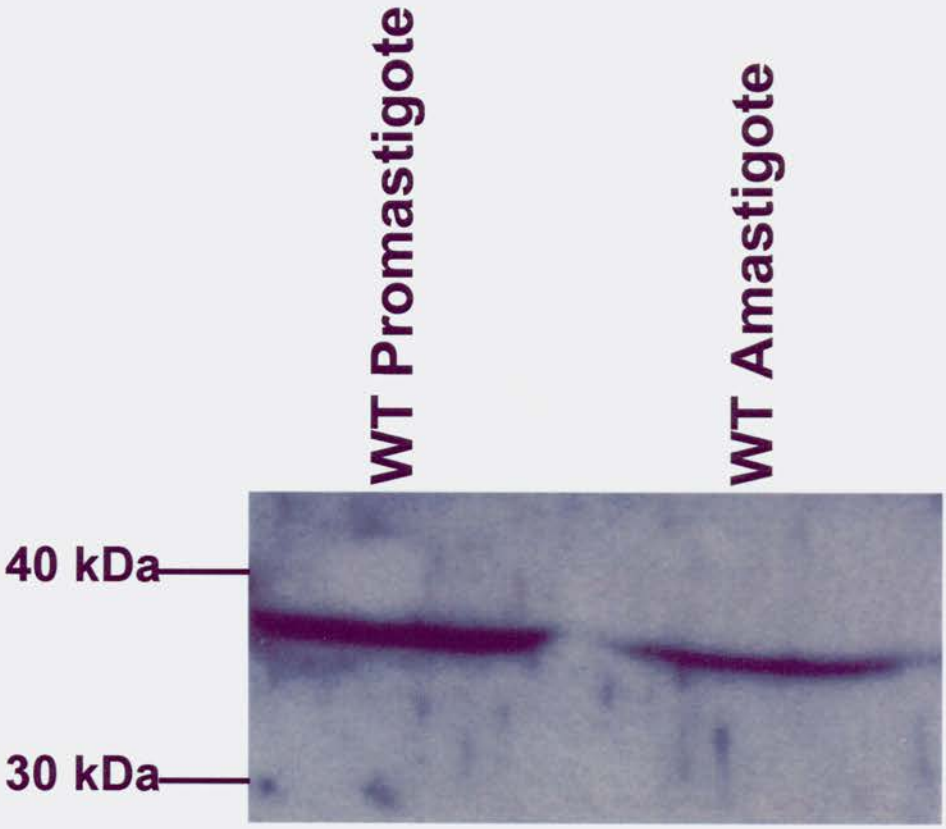

Figure 6. Western blot analysis of AAH expression in wild type promastigotes and axenic amastigotes. Lysates from wild type promastigotes and axenic amastigotes were analyzed by western blotting using polyclonal anti-sera specific for AAH.

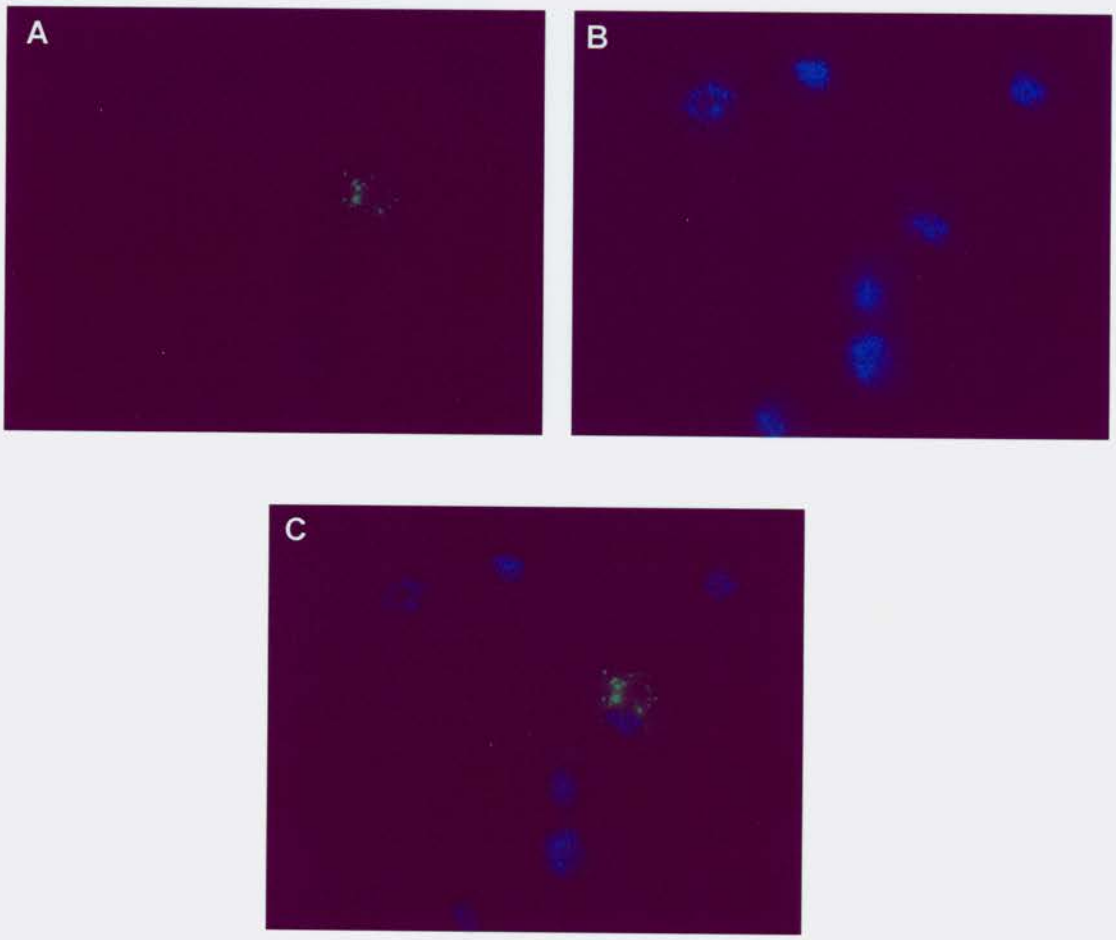

Figure 7. Immunofluorescence detection of LdAAH in intracellular amastigotes. The expression of AAH was detected using IFA with purified anti-AAH antisera. Anti-AAH antibody conjugated to Oregon Green secondary is depicted in panel A, parasite and macrophage DNA is stained with DAPI (Panel B) and the two images are overplayed (Panel C).

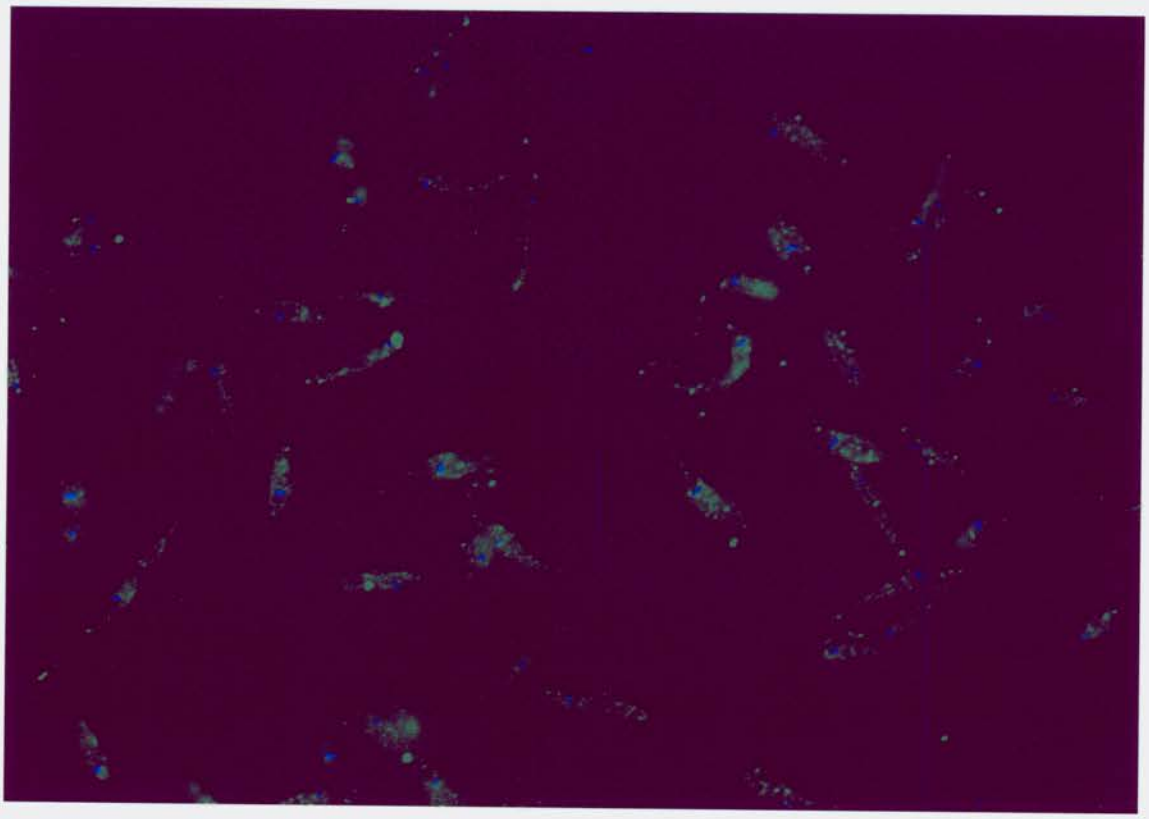

Figure 7D. Preliminary localization of LdAAH. Immunofluorescence detection of LdAAH using purified anti-AAH antisera.

3.A.6. References

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Chapter 4

Manuscript 3*

Genetic validation of the purine salvage pathway of *Leishmania donovani*

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Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AK, adenosine kinase; dCF, 2'-deoxycoformycin; AAH, adenine aminohydrolase; PBS, phosphate buffered saline; UTR, untranslated region; ORF, open reading frame; PCR, polymerase chain reaction; FBS, fetal bovine serum.

4.1. Abstract

Leishmania donovani cannot synthesize purines *de novo* and express a multiplicity of enzymes that enable them to salvage purines from their hosts. Previous efforts to generate an *L. donovani* strain deficient in both hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and xanthine phosphoribosyltransferase (XPRT) using gene replacement approaches were not successful, lending indirect support to the hypothesis that either HGPRT or XPRT is crucial for purine salvage by the parasite. We now report the genetic confirmation of this hypothesis through the construction of a conditional *Δhgprt/Δxprt* mutant strain that exhibits an absolute requirement for 2'-deoxycoformycin, an inhibitor of the leishmanial adenine aminohydrolase (AAH) enzyme, and either adenine or adenosine as a source of purine. Unlike wild type parasites, the *Δhgprt/Δxprt* strain cannot proliferate without 2'-deoxycoformycin or with hypoxanthine, guanine, xanthine, guanosine, or xanthosine as the sole purine nutrient but surprisingly can grow modestly on inosine. The *Δhgprt/Δxprt* mutant infects murine bone marrow-derived macrophages <5% as effectively as wild type parasites and cannot sustain an infection. These data establish genetically that either HGPRT or XPRT is absolutely essential for purine acquisition, parasite viability, and parasite infectivity of mouse macrophages, that all exogenous purines are funneled to hypoxanthine and/or xanthine by *L. donovani*, and that the purine sources

within the macrophage to which the parasites have access are HGPRT or XPRT substrates.

4.2. Introduction

Leishmania donovani is a protozoan parasite that is the etiologic agent of visceral leishmaniasis, a devastating and often fatal disease in humans. *Leishmania spp.* are digenetic, existing in both insect vector and mammalian forms. The flagellated, motile, extracellular promastigote proliferates in the midgut of phlebotomine sandfly family members, whereas the nonflagellated, nonmotile, intracellular amastigote resides in phagolysosomes of macrophages and other reticuloendothelial cells within the vertebrate host. Due to the absence of effective vaccines, chemotherapy has offered the only avenue of defense for the treatment and prevention of leishmaniasis and other parasitic diseases. Unfortunately, drug therapy for leishmaniasis is compromised by toxicity, expense, prolonged and invasive routes of administration, and resistance. Thus, the need for new and more efficacious drugs is acute.

The institution of an effective parasite-specific therapeutic regimen for the treatment of leishmaniasis, or for that matter any parasitic disease, depends upon exploiting fundamental biochemical or metabolic differences between parasite and host. Perhaps the most striking metabolic disparity between parasites and their mammalian hosts is the avenue by which they synthesize purine nucleotides. Whereas mammalian cells can generate the purine ring *de novo*, all of the protozoan parasites studied to date are incapable of synthesizing the purine ring (1). As a consequence, each genus of parasite has evolved a unique complement of purine salvage enzymes that enables it to scavenge host

purines (1, 2). *Leishmania* expresses a number of purine salvage enzymes. These enzymes include hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), xanthine phosphoribosyltransferase (XPRT), and adenosine kinase (AK), and the genes encoding all four *L. donovani* proteins have been isolated and sequenced (3-6). The parasite also accommodates a variety of purine interconversion enzymes including nucleosidases (1, 7), IMP branchpoint enzymes, phosphorylases, and deaminases (1, 8). Thus, the purine salvage pathway of *Leishmania* is divagating and redundant, and this metabolic complexity, as well as the diploid nature of the parasite, has hindered a thorough characterization of the pathway.

The ability of *Leishmania* to carry out efficient homologous gene replacement (9, 10) and take up foreign DNAs (11), however, can overcome these impediments and enables the genetic dissection of complex metabolic pathways such as that for purine acquisition. Implementing targeted gene replacement strategies, *L. donovani* promastigotes deficient in HGPRT, APRT, XPRT, and/or AK were created in almost every conceivable combination (5, 12, 13), although it was not possible to create a *Δhgprt/Δxprt* double mutant in any genetic background. These genetic studies underscored our central hypothesis governing purine metabolism that either HGPRT or XPRT is both necessary and sufficient for all of purine acquisition by *L. donovani*. The inability to create the *Δhgprt/Δxprt* double knockout, however, was negative evidence that did not confirm the premise.

We have now isolated and characterized a conditional *Δhgprt/Δxprt* line of *L. donovani* that was selected by targeted gene replacement in the presence of 2'-deoxycoformycin (dCF), an inhibitor of the leishmanial adenine aminohydrolase (AAH) enzyme (8), and adenine as a source of purine. The creation and characterization of this *Δhgprt/Δxprt* mutant confirms our main supposition that either HGPRT or XPRT is absolutely essential for purine acquisition and parasite viability.

4.3. Materials and Methods

4.3.1. Materials

[8-^{14}C]adenine (50 mCi/mmol), [8-^{14}C]adenosine (53 mCi/mmol), [8-^{14}C]guanine (55 mCi/mmol), [8-^{14}C]guanosine (50 mCi/mmol), [8-^{14}C]hypoxanthine (51 mCi/mmol), [8-^{14}C]inosine (52 mCi/mmol), and [8-^{14}C]xanthine (53 mCi/mmol) were all purchased from Moravek Biochemicals (Brea, CA). dCF was obtained from the National Cancer Institute (Bethesda, MD). All restriction and DNA modifying enzymes and all other chemicals and reagents were of the highest quality commercially available.

4.3.2. Axenic parasite cell culture

The *L. donovani* strain 1S2D originated with Dr. Dennis Dwyer (NIH) and was adapted for growth as axenic amastigotes as described (14). A clonal derivative of this strain, LdBob (14), was provided by Dr. Stephen Beverley (Washington University, St. Louis, MO). LdBob promastigotes were cultured at 26 °C in purine-replete M199-based medium as detailed (14) or in a modified DME-L medium (15) that lacks bovine serum albumin and is supplemented with 10% dialyzed fetal bovine serum (FBS), 1 mM glutamine, 1 X RPMI 1640 vitamin mix, 10 μM folate, 100 μM adenine, and 20 μM dCF. Axenic amastigotes were cultured at 37 °C as described (14). The characterization of LdBob strains harboring single *Δhgprt, Δaprt,* or *Δxprt* lesions is currently under review. Parasite growth experiments were seeded at 5X10^4 parasites/ml for

promastigotes and $5X10^3$ parasites/ml for axenic amastigotes and were enumerated by hemacytometer.

4.3.3. Targeting and episomal constructs

The flanking regions from the *HGPRT*, *APRT*, and *XPRT* loci and the oligonucleotides used for their amplification by the polymerase chain reaction (PCR) have been described (5, 12). The construction and authentication of the pX63-HYG-*Δhgprt*, pX63-NEO-*Δxprt*, and pX63-HYG-*Δxprt* targeting vectors employed in the previous allelic replacements of the *HGPRT* and *XPRT* loci have also been described (5, 12). The pX63-NEO-*Δhgprt/Δxprt* construct was created by replacing the 5' untranslated region (UTR) of *XPRT* in the pX63-NEO-*Δxprt* vector with the 5' UTR of the *HGPRT*. The pX63-PHLEO-*Δxprt* replacement plasmid was generated by excising the 5' and 3' UTRs of *XPRT* from pX63-HYG-*Δxprt* and inserting them into the appropriate sites within pX63-PHLEO (16).

To generate episomal constructs of *HGPRT* and *XPRT*, the two genes were amplified by PCR and inserted into the SmaI-BamHI restriction sites within the pXG-BSD blasticidin resistance expression plasmid generously provided by Dr. Stephen Beverley. The complementation vectors were designated pXG-BSD-*HGPRT* and pXG-BSD-*XPRT* respectively. The other two episomal constructs, pSNBR-HYG-*hgprtΔ(209-211)* and pXG-BSD-*xprtΔAKL* which produce cytosolic hgprt and xprt, respectively, were generated as described (17, 18). pSNBR-HYG-*hgprtΔ(209-211)* will now be referred to as pSNBR-HYG-*hgprtΔSKV*.

4.3.4. Gene replacements and complemented lines

All genetic manipulations were conducted on LdBob promastigotes. Single *Δhgprt*, *Δaprt*, and *Δxprt* knockouts were created in LdBob using the same targeting constructs, protocols, and gene replacement strategies employed previously for the generation of these mutants within an avirulent *L. donovani* background (5, 12). The *Δhgprt/Δxprt* double knockout was generated by 3 sequential rounds of targeted gene replacement. The pX63-NEO-*Δhgprt/Δxprt*, pX63-HYG-*Δhgprt*, and pX63-PHLEO-*Δxprt* plasmids were linearized with HindIII and BglII and transfected into $5X10^7$ parasites using reported electroporation conditions (14). Homologous integrations were selected by plating parasites on semi-solid medium containing selective concentrations of either Geneticin, hygromycin, or phleomycin (16), as appropriate for the drug resistance marker of the targeting cassette. The *HGPRT/hgprt/XPRT/xprt* and *Δhgprt/XPRT/xprt* lines were generated and maintained in the M199-based medium, while the *Δhgprt/Δxprt* null mutant was selected and maintained in the modified DME-L medium described above. The *HGPRT/hgprt/XPRT/xprt*, *Δhgprt/XPRT/xprt*, and *Δhgprt/Δxprt* lines were all maintained continuously under selective pressure in the drugs for which they contained resistance markers.

The *Δhgprt/Δxprt* line was also transfected separately with pXG-BSD-*HGPRT*, pXG-BSD-*XPRT* or pXG-BSD-*xprtΔAKL* and transfectants selected in 20 μg/ml blasticidin and either 100 μM hypoxanthine or 100 μM xanthine to generate the complemented lines *Δhgprt/Δxprt*[pXG-BSD-*HGPRT*], *Δhgprt/Δxprt*[pXG-BSD-*XPRT*] and *Δhgprt/Δxprt*[pXG-BSD-*xprtΔAKL*],

respectively. These cell lines are designated *Δhgprt/Δxprt*[p*XPRT*], *Δhgprt/Δxprt*[pHGPRT] and *Δhgprt/Δxprt*[p*xprtΔAKL*]. The *Δhgprt/Δxprt* cell line that was transfected with pSNBR-HYG-*hgprtΔSKV* was selected in 50 μg/ml of hygromycin and 100 μM hypoxanthine and is designated *Δhgprt/Δxprt*[p*hgprtΔSKV*].

4.3.5. DNA manipulations and Western blotting

Isolation of genomic DNA and Southern blotting were performed by conventional protocols (19). Monospecific antibodies to purified recombinant *L. donovani* HGPRT, APRT, and XPRT proteins have been described (3-5) and Western blotting protocols were carried out as conveyed (19). Mouse monoclonal antibody to the amastigote-specific A2 protein (20) was generously provided by Dr. Greg Matlashewski of McGill University Faculty of Medicine, Montreal, Quebec, Canada.

4.3.6. Enzyme assays

$2X10^9$ parasites were washed two times in phosphate buffered saline (PBS), resuspended in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM phosphoribosylpyrophosphate, and protease inhibitor cocktail and fractionated by centrifugation at 13,000 rpm at 4°C. Radiolabel incorporation assays were performed on cell-free lysates of promastigote extracts as described (21), while amastigote enzymatic assays were conducted with lysates that were not fractionated by centrifugation.

4.3.7. Purine metabolism in live cells

The rates of conversion of radiolabelled purine into nucleotides were measured using the earlier described DE-81 filter disk method (21). Cells were washed with PBS and resuspended at a density of $1X10^8$ cells/ml in either promastigote or amastigote media containing 2 μM radiolabeled purine but lacking bovine serum albumin, FBS, and hemin,. At each time-point $1X10^7$ cells were removed, washed once in PBS, lysed in 1% Triton X-100 and spotted onto DE-81 filter disks. Parasite metabolism of various radiolabeled purines was quantified by liquid scintillation.

4.3.8. Growth phenotypes

To assess the abilities of genetically manipulated strains to grow in various different purine sources, all parasites were washed several times with PBS and resuspended in modified DME-L medium lacking purine and incubated at 26°C for 4 hours before they were seeded at a density of $5X10^4$ cells/ml in 1.0 ml aliquots of modified DME-L containing 100 μM purine and 5% dialyzed FBS. Amastigotes were seeded at a density of $5X10^3$ cells/ml into amastigote media containing 20% FBS and 100 μM purine, incubated for 7-10 days, and counted by hemacytometer.

4.3.9. Macrophage infections

Promastigotes were washed two times in purine-free promastigote media and resuspended in Dulbecco's Modified Eagle medium supplemented with 4

mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% FBS. $2X10^5$ bone marrow-derived mouse macrophages, from Balb/c mice, and $2X10^6$ promastigotes were placed in 4-well Lab-TekII Chamber Slides (Nalge Nunc International Corp., Naperville, IL) containing 1.0 ml DME-L medium supplemented with 10% fetal calf serum and incubated at 37 °C in a humidified 5% CO_2 incubator. After 16 hr, adherent macrophages were washed ten times in PBS to eliminate residual extracellular promastigotes after which fresh growth medium was added and then again 24 hours later. After an additional 24 hour incubation, the chambers were washed three times with PBS and macrophages were stained using the Diff-Quik kit (International Medical Equipment Inc., San Marcos, CA). Parasites were visualized on a Zeiss Axiovert 200M scope (Carl Zeiss Microimaging, Thornwood, NY) using 60X oil immersion light and photographed with an AxioCam MRm camera (Zeiss) and parasites enumerated. Color photographs of parasitized macrophages were visualized on a Zeiss Axiophot microscope using 40X oil immersion and photographed with a Leica DC 300 camera (Leica Camera AG, Solms, Germany).

4.4. Results

4.4.1. Creation of the *Δhgprt/Δxprt* line by gene replacement

The *Δhgprt/Δxprt* knockout was created after three rounds of transfection with drug resistance cassettes carrying 5' and 3' UTRs of *HGPRT* and *XPRT* (Fig. 1). Because *HGPRT* and *XPRT* are colocalized within a 4359 bp EcoRI fragment in the *L. donovani* genome (5), the first copy of both genes was displaced with X63-NEO-*Δhgprt/Δxprt* (linearized pX63-NEO-*Δhgprt/Δxprt*), a construct containing the 5' UTR of *HGPRT* and the 3' UTR of *XPRT*, to create the *HGPRT/hgprt/XPRT/xprt* double heterozygote. The heterozygote was then transfected with X63-HYG-*Δhgprt* to generate the *Δhgprt/XPRT/xprt* line, and the latter was transfected with X63-PHLEO-*Δxprt* to create the *Δhgprt/Δxprt* double knockout (Fig. 1). The last round of transfection was performed in medium containing 20 μM dCF and 100 μM adenine, while the *HGPRT/hgprt/XPRT/xprt* and *Δhgprt/XPRT/xprt* progenitors of the double knockout were isolated in medium lacking dCF and containing 100 μM adenine as a purine source. 50-100 drug-resistant colonies were obtained within days after the first two cycles of transfections, but surprisingly only two barely visible colonies were obtained after four weeks following the last round of transfection in the adenine-dCF medium. Both colonies were picked and expanded in liquid culture medium containing adenine and dCF.

Southern blot analysis of the *HGPRT/hgprt/XPRT/xprt*, *Δhgprt/XPRT/xprt*, and *Δhgprt/Δxprt* strains divulged the new alleles that had been created by the

homologous gene replacement events (Fig. 2). Digestion of genomic DNA with EcoRI and hybridization to the *HGPRT* and *XPRT* ORFs revealed the presence of the common 4359 bp restriction fragment in the wild type and *HGPRT/hgprt/XPRT/xprt* lines and its absence in the *Δhgprt/Δxprt* null mutant. The *Δhgprt/XPRT/xprt* strain, as expected, lacks a band for HGPRT but exhibits a hybridization signal at 5939 bp when probed with the *XPRT* ORF that reflects the appropriate integration of the hygromycin resistance marker within the X63-HYG-*Δhgprt* cassette into the *HGPRT* locus. For comparison, Southern blot analysis of previously isolated single knockouts of HGPRT, APRT, and XPRT are also depicted, and the signals are appropriate for the expected homologous integrations (Fig. 2).

4.4.2. Western blot analysis of phosphoribosyltransferases in knockout parasites

Western blot analysis of wild type, *HGPRT/hgprt/XPRT/xprt*, *Δhgprt/XPRT/xprt,* and *Δhgprt/Δxprt* extracts confirmed the absence of HGPRT and XPRT protein in strains in which the corresponding gene had been eliminated (Fig. 3). The single *Δhgprt*, *Δaprt*, and *Δxprt* null mutants for which Southern blot data are shown in Fig. 2 also lacked the proteins corresponding to the deleted genes.

4.4.3. Enzyme assays in promastigote extracts

The abilities of wild type and *Δhgprt/Δxprt* promastigote lysates to incorporate individual radiolabelled purines into nucleotides were assessed over a 15 min time interval (Fig. 4). Whereas wild type promastigotes were capable of incorporating all purine bases and nucleosides tested, the *Δhgprt/Δxprt* could not convert guanine, guanosine, hypoxanthine, or inosine to nucleotides. The double knockout could, however, incorporate radiolabeled adenine and adenosine into phosphorylated products during the 15 min assay interval, whether or not dCF was added to the extracts (Fig. 4). [^{14}C]Xanthine conversion to nucleotides could not be measured in promastigote extracts for technical reasons.

4.4.4. Purine metabolism in intact promastigotes

The ability of live promastigotes to metabolize various [^{14}C] radiolabeled purine bases and nucleosides was also measured. These results corroborated the results from the enzymatic assays described above. Additionally, the metabolism of [^{14}C]xanthine could be measured in intact wild type cells but not in the double knockout (Fig. 5). [^{14}C]Adenine and [^{14}C]adenosine metabolism could be measured in both wild type and mutant cells during the two hour time-course in the presence or absence of dCF.

4.4.5. Growth phenotype of *Δhgprt/Δxprt* promastigotes

The ability of *Δhgprt/Δxprt* promastigotes to proliferate in various purine sources was assessed (Fig. 6). Whereas wild type parasites could grow in modified DME-L medium with any of the added purine nucleobases or

nucleosides as a purine source, continuous growth of *Δhgprt/Δxprt* promastigotes could be maintained only in adenine or adenosine in the presence of 20 μM dCF. The double mutant could not grow in xanthine, xanthosine, guanine, guanosine, or hypoxanthine, although a limited amount of growth was observed when inosine was added as the purine. The limited proliferation in inosine was further investigated with immucillin H, an iminoribitol analog of inosine that is a potent inhibitor of the leishmanial inosine-uridine nucleoside hydrolase activity (22). Immucillin H at 20 μM enabled robust growth of the *Δhgprt/Δxprt* double knockout in 100 μM inosine (data not shown).

4.4.6. Complementation of the *Δhgprt/Δxprt* knockout

The *Δhgprt/Δxprt* transfectants *Δhgprt/Δxprt*[p*HGPRT*] and *Δhgprt/Δxprt*[p*XPRT*] exhibited the same growth phenotypes as the *Δxprt* and *Δhgprt* single mutants, respectively; *Δhgprt/Δxprt*[p*XPRT*] parasites could grow on all purines, whereas *Δhgprt/Δxprt*[p*HGPRT*] could grow on all purines except guanine, xanthine and xanthosine (data not shown). dCF was not necessary for growth with adenine or adenosine in the complemented lines. Complementation of the *Δhgprt/Δxprt* line could also be achieved with episomes expressing cytosolic versions of hgprt and xprt. The *Δhgprt/Δxprt*[p*hgprtΔSKV*] and *Δhgprt/Δxprt*[p*xprtΔAKL*] cell lines grew robustly in either hypoxanthine or xanthine.

4.4.7. Characterization of *Δhgprt/Δxprt* axenic amastigotes

Both wild type and *Δhgprt/Δxprt* parasites were capable of transformation to axenic amastigotes, as assessed by their expression of A2 proteins, a family of amastigote-specific markers (23). No A2 was observed in the promastigotes. The growth phenotypes of both wild type and knockout axenic amastigotes were identical to their promastigote counterparts (data not shown). The metabolic capacities of the axenic amastigotes toward sundry purines were also indistinguishable from the promastigote equivalent.

4.4.8. Macrophage infections

Wild type *L. donovani* promastigotes were capable of sustaining a robust infection of bone marrow-derived murine macrophages, whereas the *Δhgprt/Δxprt* knockout could not (Fig. 7). Parasitemia of the wild type strain was ~21 parasites/macrophage, while the double knockout infectivity was ~1 parasite/macrophage, a 20-fold difference. The inability of *Δhgprt/Δxprt* cells to proliferate inside macrophages could not be imputed to a failure to infect the mammalian cells, because similar numbers of intracellular parasites were observed for both wild type and knockout parasites 4 hr post-infection (data not shown). Both complemented lines, *Δhgprt/Δxprt*[p*HGPRT*] and *Δhgprt/Δxprt*[pX*PRT*], also sustained robust infections in the macrophages, although the infectivity was lower than that of wild type parasites. Infectivity levels of ~8 parasites/macrophage and ~13 parasites/macrophage were observed for *Δhgprt/Δxprt*[p*HGPRT*] and *Δhgprt/Δxprt*[pX*PRT*], respectively (data not shown).

4.5. Discussion

The purine salvage pathway of *Leishmania* is myriad and complex, although determining which of the many routes of purine salvage are functional has defied genetic dissection (1, 24). Mutational and gene replacement schemes in *L. donovani* have demonstrated that none of the four known enzymes capable of converting host purine nucleobases or nucleosides to the nucleotide level, HGPRT, APRT, XPRT, or AK, is essential by itself (5, 12, 13, 25). Furthermore, the ability to generate viable *Δhgprt/Δaprt/ak*$^-$ (13) and *Δxprt/Δaprt/ak*$^-$ (unpublished) *L. donovani* promastigotes reveals that the parasite can rely on either a functional XPRT or HGPRT activity for all of its purine nutritional requirements. Indeed, it has been possible to generate mutant parasites by targeted gene replacement in every conceivable combination except mutants accommodating a combined *Δhgprt* and *Δxprt* genotype. These results were the basis for our fundamental hypothesis that either HGPRT or XPRT is necessary and sufficient for *Leishmania* parasites to salvage purines, maintain viability, and sustain proliferation. The hypothesis was sustained by the inability to generate a *Δhgprt/Δxprt* double knockout even within a genetic background complemented with an episomal copy of either *HGPRT* or *XPRT* (unpublished results).

We now report genetic proof of our principal hypothesis by the creation and characterization of a conditional *Δhgprt/Δxprt* double knockout. Taking advantage of the co-localization of the *HGPRT* and *XPRT* genes in the leishmanial genome (5), a *Δhgprt/Δxprt* mutant was selected after three rounds of targeted gene replacement, with the final transfection being achieved in the

presence of 100 μM adenine and 20 μM dCF, an inhibitor of the *L. donovani* AAH (8). The *Δhgprt/Δxprt* mutant is absolutely reliant on the presence of dCF and either adenine or adenosine as a purine source. No other naturally occurring purine tested could enable sustained proliferation of either stage of the parasite, except inosine when provided with immucillin H, an inhibitor of the inosine-uridine nucleoside hydrolase activity of *L. donovani* (22). The ability of *Δhgprt/Δxprt* promastigotes to grow in inosine and immucillin H suggests that *L. donovani* express an activity capable of salvaging the nucleoside. Although inosine kinase activity has not been detected in *Leishmania* (1), a nucleoside phosphotransferase activity capable of phosphorylating the pyrazolopyrimidine nucleoside analogs allopurinol riboside and formycin B, has been observed in *L. donovani* promastigotes (26, 27). Whether this phosphotransferase is also capable of recognizing inosine is not known.

To date, there are no effective vaccines to protect against visceral leishmaniasis (28). Generating a strain with intrinsic attenuating mutation(s) could theoretically be exploited as a live attenuated vaccine for immunizing against the disease is a valid alternative strategy to control leishmaniasis rather than the conventional paradigm of treatment with toxic drugs. Previous studies using non-infectious *L. tarentolae* or attenuated strains of *L. major*, have demonstrated that these lines are capable of triggering a protective immune response against further challenge by virulent *Leishmania* in susceptible rodents (28, 29). The inability of the *Δhgprt/Δxprt* knockout to sustain an infection of

murine macrophages bolsters this mutant strain as a candidate for such a live vaccine strategy against visceral leishmaniasis. The *Δhgprt/Δxprt* mutant infects macrophages at a level < 5% of that of wild type parasites. Preliminary results indicate that the overall infection rate of the *Δhgprt/Δxprt* mutant in macrophages can be increased by the addition of either adenine alone or, a combination of adenine and dCF, to the growth medium. Thus, it should be theoretically feasible to sustain an infection of the *Δhgprt/Δxprt* strain in susceptible mammals by dietary supplementation with adenine and/or dCF until a protective immune response has been established. The strain could then be eliminated by withdrawal of the dietary additions.

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4.6 Figures

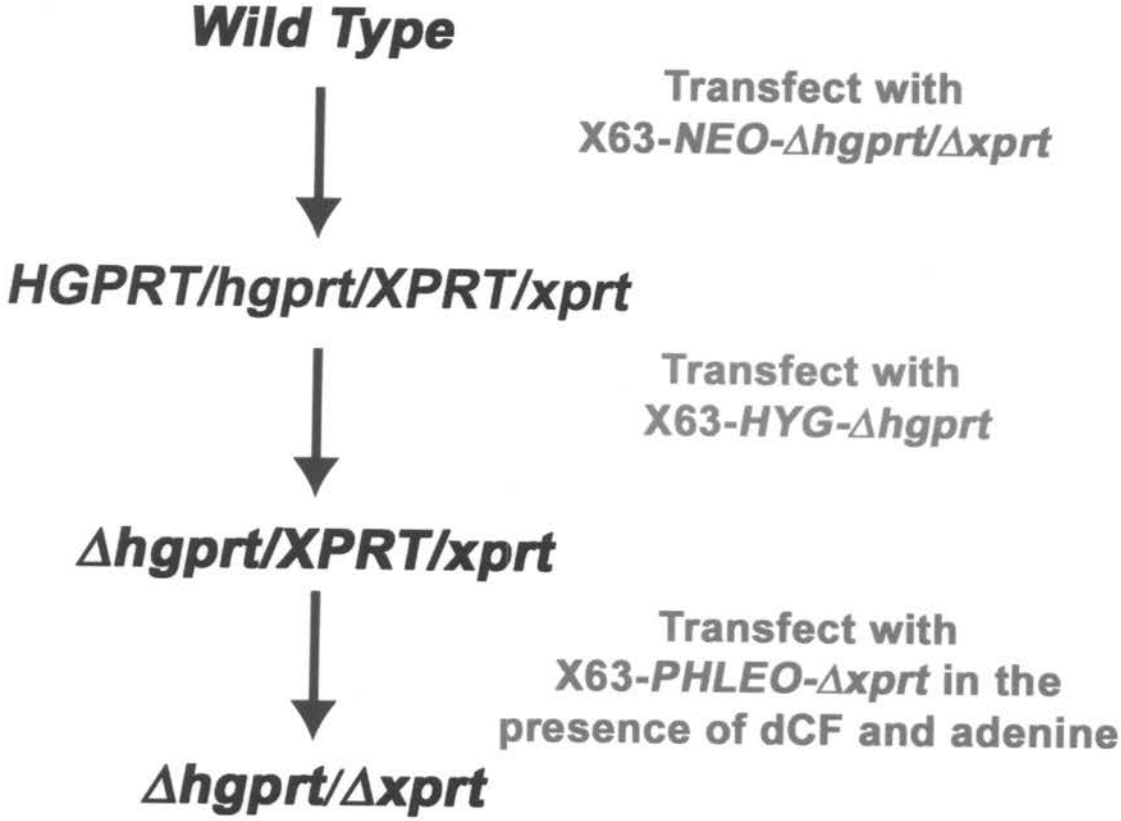

Figure 1. Strategy for the creation of the *Δhgprt/Δxprt* cell line. The *Δhgprt/Δxprt* double knockout was created after three rounds of transfection with the indicated targeting constructs. The cell line nomenclature is described in Materials and Methods. The *HGPRT/hgprt/XPRT/xprt* heterozygote and *Δhgprt/XPRT/xprt* lines were selected in 100 μM adenine and the *Δhgprt/Δxprt* null mutant was isolated in semi-solid medium containing 100 μM adenine and 20 μM dCF.

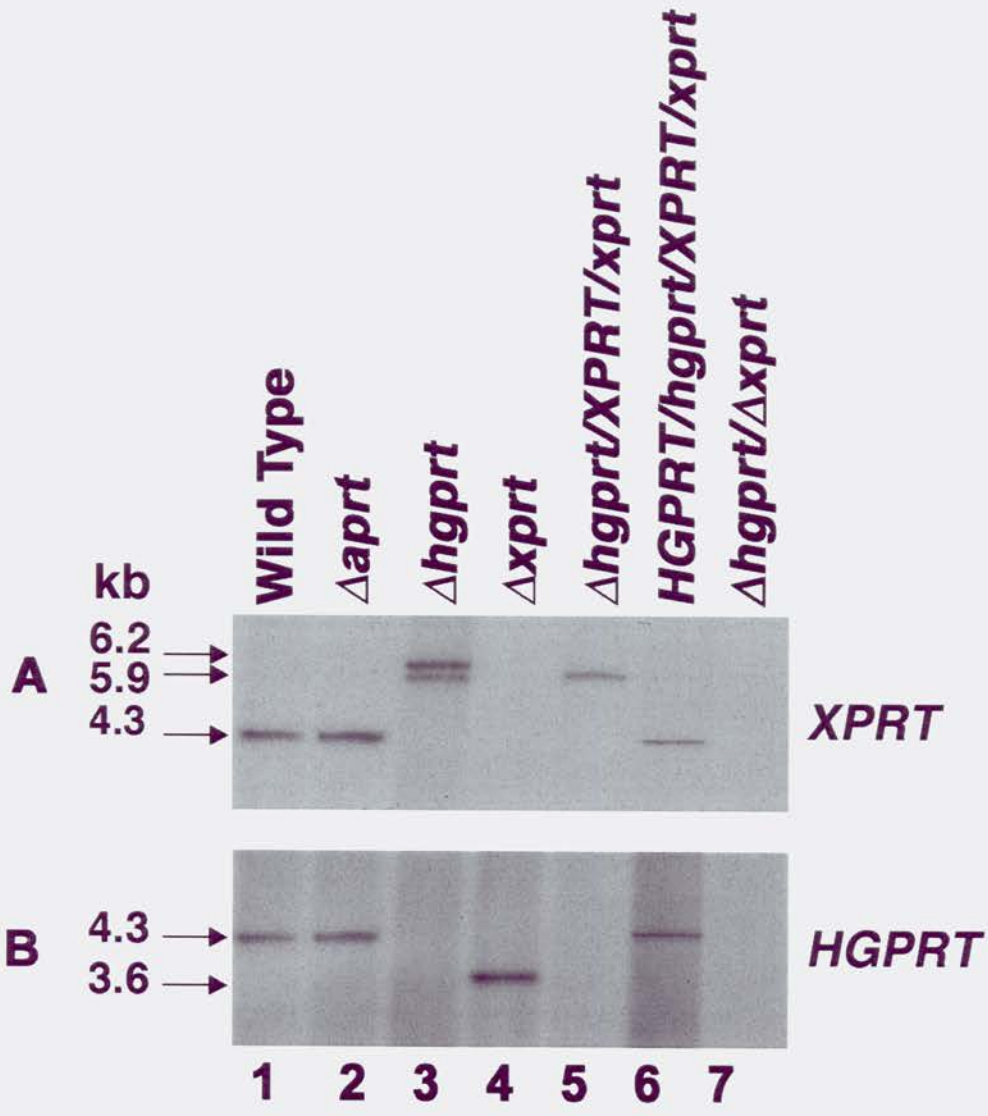

Figure 2. Southern blot analysis of the *Δhgprt/Δxprt* mutant. Genomic DNA from wild type, *Δaprt*, *Δhgprt*, *Δxprt*, *Δhgprt/XPRT/xprt*, *HGPRT/hgprt/XPRT/xprt*, and *Δhgprt/Δxprt* parasites was digested with EcoRI, fractionated on 0.8% agarose and blotted onto a nylon membrane. The blot was hybridized under high stringency conditions and probed first with the full-length *XPRT* ORF (A). The blot was then stripped and probed with the full-length *HGPRT* ORF (B).

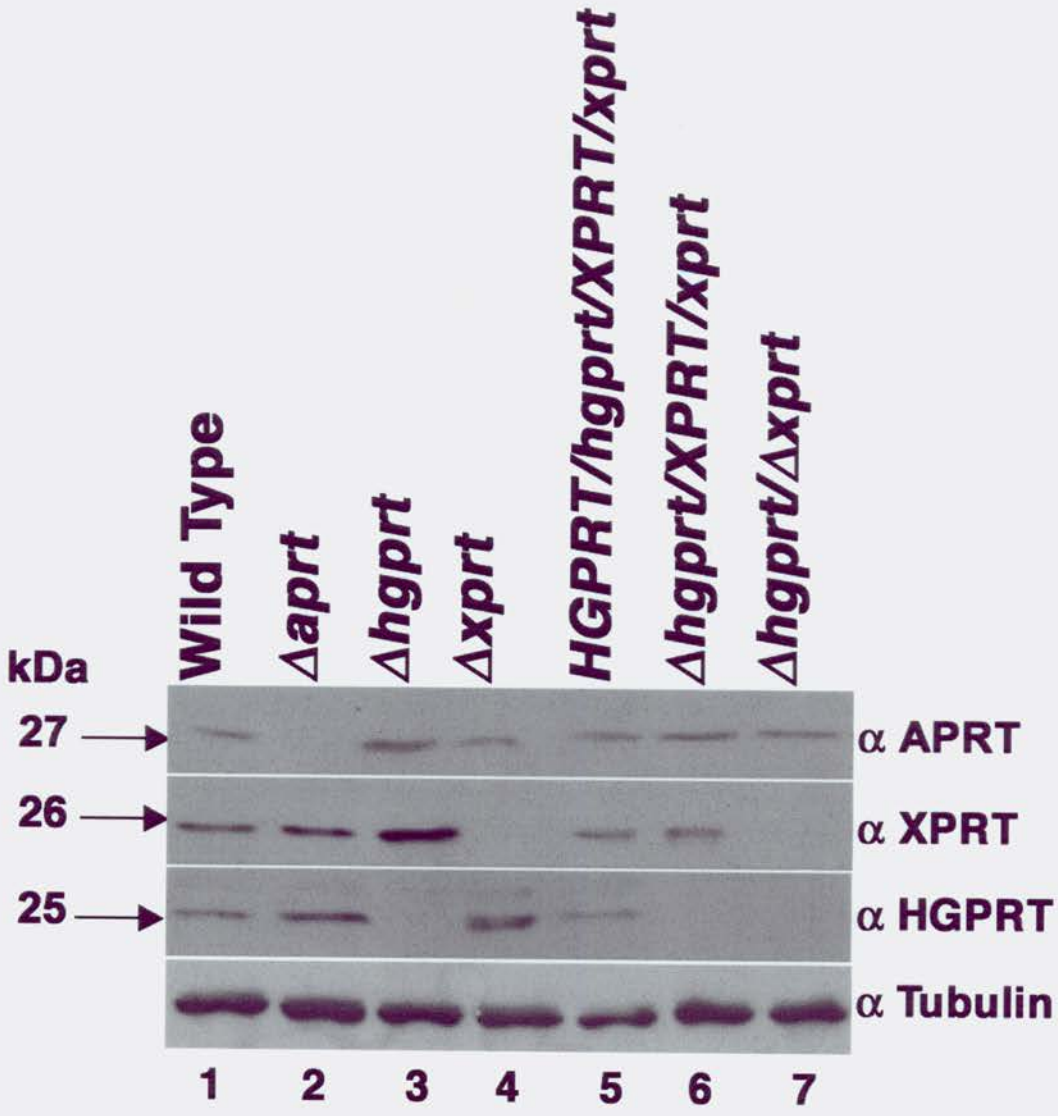

Figure 3. Western blot analysis of the *Δhgprt/Δxprt* mutant. Lysates of exponentially growing wild type and mutant parasites were analyzed by immunoblotting using anti-APRT, anti-HGPRT, and anti-XPRT monospecific polyclonal antisera. The amount of protein loaded onto each lane was normalized by blotting with tubulin antisera.

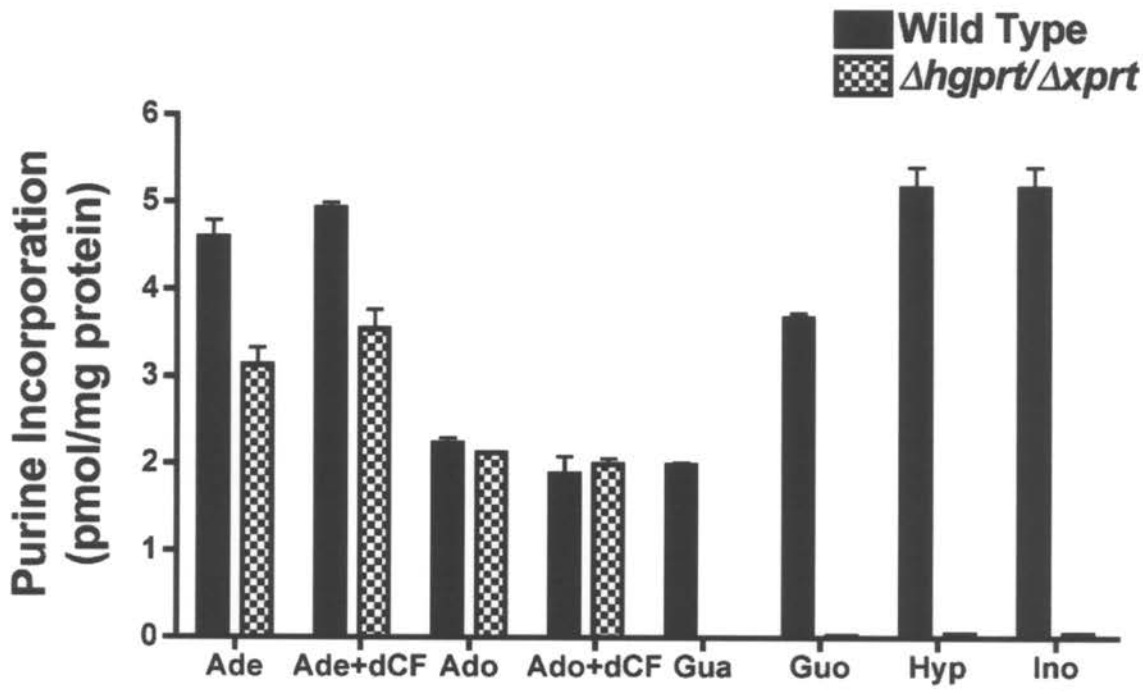

Figure 4. Enzyme assays in promastigote extracts. The ability of extracts from wild type and *Δhgprt/Δxprt* parasites to incorporate indicated [^{14}C] purines into phosphorylated products was measured over a 15 min time-course as described in "Materials and Methods".

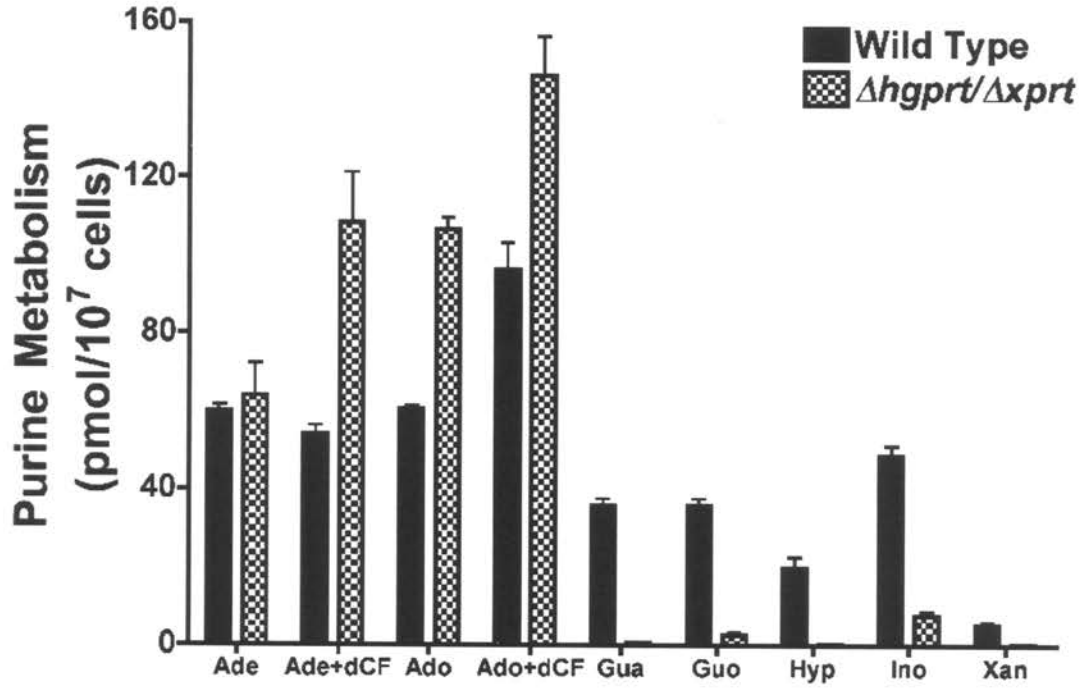

Figure 5. Purine metabolism in intact promastigotes. The capacity of live wild type and *Δhgprt/Δxprt* parasites to metabolize radiolabeled purines into phosphorylated products was assessed over a 2 hour time course.

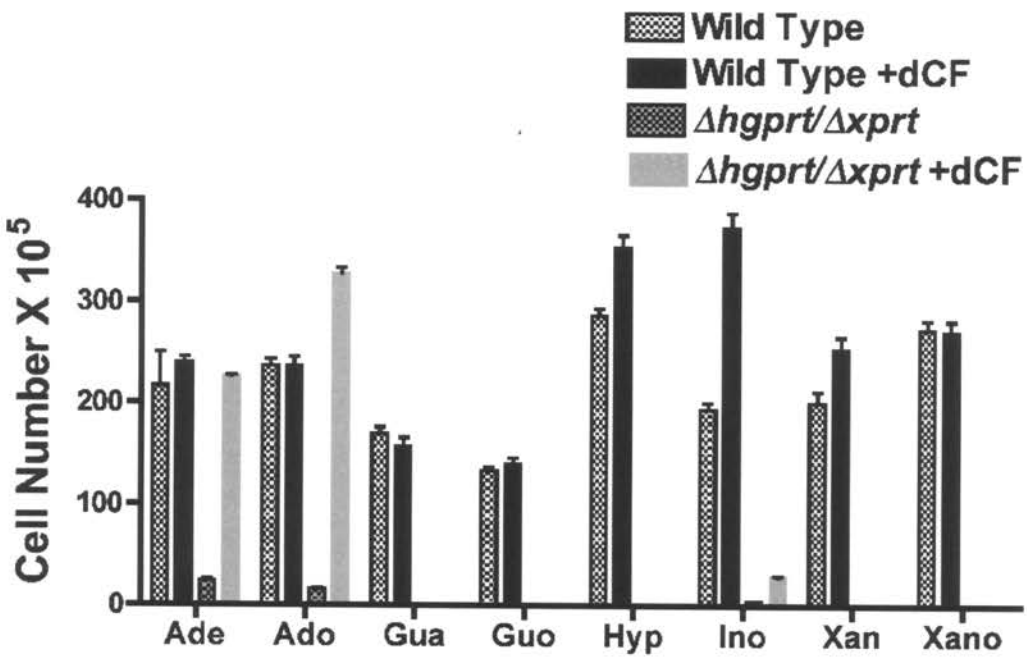

Figure 6. Growth of promastigotes in various purine sources. The ability of wild type parasites and the *Δhgprt/Δxprt* mutant to grow in different purine sources in the presence and absence of 20 μM dCF was investigated. Parasites were seeded at a density of $5X10^4$/mL, incubated for 7-10 days and enumerated by hemacytometer.

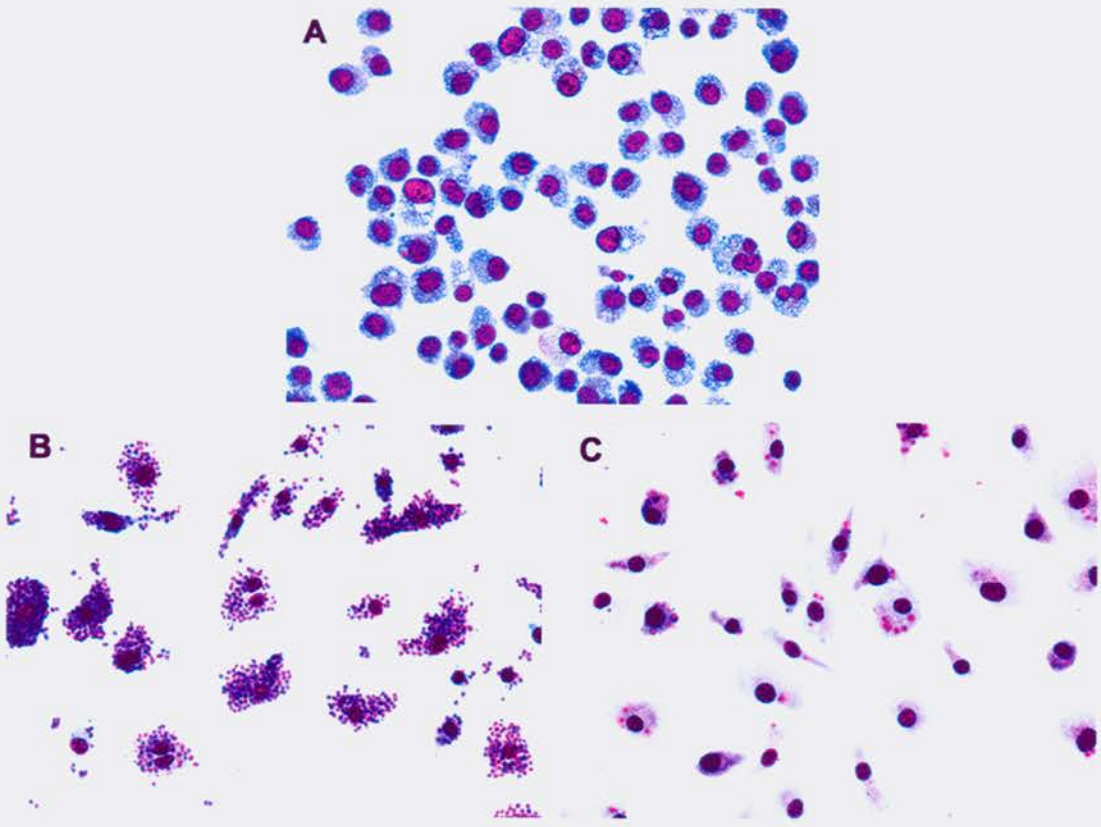

Figure 7. Infection of bone marrow-derived murine macrophages with wild type or *Δhgprt/Δxprt* parasites. Infections of bone marrow-derived macrophages were accomplished as described in "Materials and Methods". Uninfected macrophages (A) and macrophages infected with either wild type (B) or *Δhgprt/Δxprt* (C) parasites are depicted.

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Chapter 5

Conclusions and Future Directions

5.1. Conclusions Part 1

In 1978 Joseph J. Marr, Randolph Berens and Donald Nelson established that there is no *de novo* synthesis of the purine ring in *Leishmania* spp., and they proposed a salvage route for purine nucleobases and nucleosides using TLC and HPLC. Throughout the course of my thesis studies I have confirmed the existence of many of the purine salvage activities they proposed, including a nucleoside hydrolase, guanine deaminase and a phosphotransferase, through the generation and analysis of several *L. donovani* mutants that were deficient in one or more purine salvage activity. However, the gene sequences encoding many these activities still remain to be cloned and their protein products characterized in full. I have also determined that nutritional purines from the host are funneled to hypoxanthine and/or xanthine and that effectively all of the adenine and the majority of adenosine to which *L. donovani* promastigotes have access is converted ultimately to hypoxanthine, a process that requires adenine aminohydrolase (AAH). The presence of an adenine aminohydrolase in *L. donovani* promastigotes, and the idea that the preferred route of adenine metabolism is through this enzyme rather than APRT, was also proposed by Marr and Berens. The existence of AAH became most evident while analyzing adenine metabolism in extracts of *Daprt* promastigotes. In these experiments adenine was readily converted to a phosphorylated product at a level approximately half that of wild type. This conversion could be obliterated by the addition of 2'-deoxycoformycin, a drug demonstrated by G. W. Kidder and Linda

Nolan to be a potent inhibitor of adenase activity in *Leishmania* and *Crithidia fasciculada*.

Using *S. cerevisiae aah1p* as a blast query (EC 3.5.4.2), I was able to identify an *AAH* gene candidate in the *L. major* database and designed primers to amplify the putative *AAH* gene from *L. major* genomic DNA. The amplified DNA was then used as a probe to isolate the *AAH* gene from an *L. donovani* cosmid library. *L. donovani AAH* (*LdAAH*) was sequenced (EC DQ093583) and the encoded protein was found to be 35% identical with its yeast counterpart. *LdAAH* was then inserted into a bacterial expression vector containing an NH_2-terminal His_6 tag, overexpressed in *E. coli*, and tens of milligrams of soluble LdAAH protein were purified to homogeneity over a Ni^{2+}-agarose matrix. Using thin layer chromatography (TLC), I have determined that LdAAH is specific for adenine and does not metabolize adenosine.

No mammalian AAH ortholog has been identified to date. AAH is a unique enzyme whose activity has only been described in *S. cerevisiae*, *Crithidia fasciculata* and *Leishmania spp*. On a phylogenetic tree, *L. donovani* AAH clusters closest to *S. cerevisiae* AAH, and furthest from the adenosine deaminases (ADA) of human and mouse. In addition, there are no other sequences throughout any of the trypanosomatid databases that share significant homology with LdAAH, therefore its uniqueness potentially makes AAH a good drug target for the development of a subversive substrate. I would like to continue studying AAH and propose the following specific aims in order to perform a biochemical and functional analysis of this protein.

Specific Aims

I. To localize the LdAAH protein. Preliminary results indicate that LdAAH is a glycosomal protein. The LdAAH location within promastigotes and axenic amastigotes will be confirmed by indirect immunofluorescence using purified, polyclonal antiserum to LdAAH and colocalized with glycosomal LdIMPDH.

II. To characterize LdAAH biochemically. I will determine a K_m value for adenine and possibly other 6-amino-purine analogs, a K_i value for dCF, and will submit purified protein to the Structural Genomics for Pathogenic Protozoa (SGPP) consortium in Seattle to initiate crystallographic studies.

III. To insert the *Δldaah* lesion into wild type, *Δaprt* and *Δhgprt/Δxprt* parasites. Generating the *Δldaah* mutation in wild type and *Δaprt* parasites will allow a test of LdAAH function in the intact parasite and will allow me to confirm that APRT and AAH are the sole routes of adenine metabolism in *L. donovani* parasites. Inserting the *Δldaah* lesion into *Δhgprt/Δxprt* mutants should stabilize their conditionally lethal phenotype and eliminate the requirement of dCF for their survival.

5.1.2. Conclusions Part 2

The main goal of my Ph. D. thesis has been to genetically dissect the purine salvage pathway of *Leishmania donovani*, the causative agent of visceral leishmaniasis. This was done by generating and analyzing several mutant cell lines that were deficient in one or more purine salvage activities. From this work I have genetically confirmed that the majority of salvaged purines are funneled to hypoxanthine and/or xanthine and that either HGPRT or XPRT (although cytosolic hgprt and xprt are also sufficient) is necessary and sufficient for the survival of *L. donovani* promastigotes. The dependency of *L. donovani* on a hypoxanthine or xanthine metabolizing enzyme was proven through the construction of a conditional *Δhgprt/Δxprt* mutant strain that exhibits an absolute requirement for 2'-deoxycoformycin and a 6- amino purine source. Wild type, *Δaprt*, *Δhgprt* and *Δxprt* single mutants can all proliferate as axenic amastigotes and can replicate within bone marrow-derived macrophages. In contrast, *Δhgprt/Δxprt* parasites can survive as axenic amastigotes, but do not replicate within bone marrow-derived macrophages, even though their initial infection rate is equivalent to that of wild type parasites. The inability of the *Δhgprt/Δxprt* mutants to proliferate within macrophages suggests that they could be exploited as a live vaccine against visceral leishmaniasis. Because the drugs used to treat all forms of leishmaniasis are highly toxic to humans and are becoming increasingly less effective against *Leishmania* parasites, the development of anti-leishmania vaccines is a good solution for preventing new cases of leishmaniasis. I am therefore proposing to investigate the potential of

Δhgprt/Δxprt parasites to serve as a vaccine against visceral leishmaniasis. This research will be carried out in the laboratory of Dr. Buddy Ullman, Ph. D., in collaboration with Dr. Mary Wilson, M.D., University of Iowa, Iowa City, Iowa, during the duration of my post-doctoral studies.

Specific Aims

I. To determine if *L. donovani Δhgprt/Δxprt* parasites are infective in a rodent model. I will determine whether the *Δhgprt/Δxprt* parasites can proliferate and cause disease in Chinese golden hamsters mice.

II. To study the immune response in mice challenged with *L. donovani Δhgprt/Δxprt* parasites. I will determine if hamsters challenged with *Δhgprt/Δxprt* parasites display a Th1 or Th2 immune response, the former being consistent with disease curing, and the latter with disease progression, by measuring the levels of IFN-γ, IL-4, IL-10 and other cytokines produced in hamsters upon inoculation with *Δhgprt/Δxprt* parasites.

III. Determine if a challenge with *Δhgprt/Δxprt L. donovani* can immunize against subsequent re-infection with wild type *L. donovani*. If *Δhgprt/Δxprt* mutants can successfully infect a rodent model without causing disease, I will infect hamsters that have been challenged with *Δhgprt/Δxprt* parasites with wild type *L. donovani* and determine whether or not the immune response evoked

from challenge with the non-virulent parasites can protect against infection from highly virulent wild type parasites.