Molecular and Biochemical characterization of the Adenosine/Pyrimidine Nucleoside Transporter from Wild type and Drug-resistant strains of *Leishmania donovani*

by

Gayatri A. Vasudevan

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Gayatri A. Vasudevan

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Associate Dean for Graduate Studies

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Dedicated to my Parents

Abstract

Leishmania are incapable of synthesizing the purine ring *de novo* and must acquire all of their purines from their hosts. These parasites possess a unique purine salvage pathway that enables them to salvage host purines efficiently. The first step in this pathway is the transmembrane transport of preformed purine nucleosides or nucleobases mediated by specific transporters located on the parasite plasma membrane. *Leishmania donovani* promastigotes (life cycle stage within the insect host) possess two distinct nucleoside transport systems with different substrate specificities. LdNT1 mediates the uptake of adenosine and pyrimidine nucleosides as well as the cytotoxic adenosine analog tubercidin and LdNT2 transports inosine, guanosine and the toxic inosine analog formycin B.

A mutant *L. donovani* cell line deficient in LdNT1 transport activity and consequently resistant to tubercidin was previously isolated following chemical mutagenesis. This transport-deficient cell line was used to clone the *LdNT1* genes by functional rescue. Following transfection of the tubercidin-resistant TUBA5 parasites with an *L. donovani* genomic library in a cosmid vector, several transfectants were obtained with a restored sensitivity to tubercidin. Further analysis of one such cosmid clone led to the identification of two closely related genes *LdNT1.1* and *LdNT1.2* that restored both the tubercidin sensitivity and adenosine transport capability of the TUBA5 cells. Both LdNT1.1 and LdNT1.2 contained 491 amino acids and 11 predicted transmembrane segments, but differed at 6 amino acid positions. These amino acid differences conferred different kinetic properties on the two transporters. LdNT1.1 had

a higher apparent affinity for both adenosine (LdNT1.1, K_m = 0.17 μM & LdNT1.2, K_m = 0.7 μM) and uridine (LdNT1.1, K_m = 5 μM & LdNT1.2, K_m = 40 μM) compared with LdNT1.2. Interestingly, while *LdNT1.1* mRNA was abundantly expressed in both wild type and TUBA5 promastigotes, the *LdNT1.2* transcript could not be detected in either cell type. Apparently LdNT1.1 contributes virtually completely to adenosine-pyrimidine nucleoside transport in this life cycle stage of the parasite.

The tubercidin-resistant TUBA5 cell line was characterized in order to identify the molecular basis for this transport-deficient phenotype. The loss of function phenotype was neither due to large rearrangements or deletions at the *LdNT1* locus, nor due to the lack of an *LdNT1* transcript. Sequencing the *LdNT1.1* and *LdNT1.2* genes from the TUBA5 parasites revealed that while the *LdNT1.2* genes were wild type, the *LdNT1.1* genes contained single but distinct point mutations within their ORFs, indicating that the TUBA5 cells were compound heterozygotes at the LdNT1 locus. One mutant *LdNT1.1* allele encoded a transporter with a G183D substitution in predicted TM 5 and the other encoded a transporter with a C337Y substitution in predicted TM 7. Neither mutant allele, when overexpressed, could confer tubercidin sensitivity or adenosine transport capability to the TUBA5 parasites, confirming that these mutations together were responsible for the loss of function phenotype of this cell line. Kinetic analyses revealed that both mutations significantly lowered the V_{max} of transport but only increased K_m values slightly. As the mutant transporters trafficked correctly to the plasma membrane, the reduction in V_{max} plausibly resulted from impairment in the functioning of the transporter itself.

While neither G183 nor C337 was essential for adenosine transport, G183 was essential for uridine transport mediated by LdNT1.1. The mutant, G183A transported adenosine robustly but failed to take up uridine. Thus G183 is a determinant of substrate selectivity in LdNT1.1. TM 5 is distinctly amphipathic and G183 is located approximately in the center of the hydrophilic face. It is therefore conceivable that TM 5 forms part of the substrate permeation pathway through LdNT1.1.

This thesis therefore describes the functional cloning of the *LdNT1* genes, the characterization of the encoded permeases and preliminary structure-function studies on the LdNT1.1 transporter.

Chapter 1: Introduction

1. Kinetoplastids

Leishmania are single celled parasitic protozoa belonging to the order Kinetoplastida. Kinetoplastids exhibit three different lifestyles: those belonging to the family *Bodonidae* are either free living or monogenetic parasites that parasitize a single host whereas flagellates in the family *Trypanosomatidae* are either monogenetic or digenetic parasites (1). *Leishmania* and *Trypanosoma* are digenetic taxa belonging to *Trypanosomatidae* that alternate between invertebrate and vertebrate hosts. Kinetoplastids are among the most ancient eukaryotes and are believed to be evolutionarily situated on the genetic border between prokaryotes and eukaryotes (2). They possess some features in common with prokaryotes such as the lack of conventional introns and polycistronic transcription and others in common with eukaryotes including the presence of a nucleus, mitochondrion, polyadenylation of mRNA etc. Perhaps most interestingly, they possess several unique features that are not found in either prokaryotes or eukaryotes such as the presence of kinetoplast DNA, *trans*-splicing of precursor mRNA and mitochondrial RNA editing. Kinetoplastid protozoa have a single mitochondrion, which contains the mitochondrial (kinetoplast) DNA adjacent to the basal body of the flagellum (3). The kinetoplast DNA contains 20-50 catenated maxicircle molecules (23-36 kb in size) that code for rRNAs, structural genes and cryptogenes (genes with incomplete open reading frames) and 5000-12000 catenated minicircles that encode guide RNAs (gRNAs). These gRNAs are short RNAs that participate in uridine (U) insertion/deletion editing of the mitochondrial cryptogenes. Transcripts of 11 or 12 out of the 20 identified maxicircle genes are edited

to varying extents (4). This editing process corrects frameshifts, creates translation initiation and stop codons and thus determines the mature coding sequence of the mitochondrial mRNAs. Guide RNAs form anchor duplexes with pre-edited mRNAs just downstream of the sequence to be edited. The mismatch between the gRNA and the pre-edited mRNA upstream of the anchor duplex identifies the editing site and the number of uridine residues to be added or deleted (5). This process is catalyzed by a multiprotein complex called the editosome that contains the RNA endonuclease, terminal uridylyl transferase, 3'U-specific exonuclease and RNA ligase activities (5).

Another feature unique to kinetoplastids is the *trans*-splicing of a 39-nucleotide leader sequence (spliced leader) onto the 5' ends of all nuclear mRNAs. Most kinetoplastid genes are transcribed into large polycistronic precursor RNAs that are subsequently cleaved into monocistronic mRNAs by the the action of two intergenic RNA cleaving reactions, *trans*-splicing to create the 5' ends and 3' cleavage and polyadenylation to generate mature 3' ends (2). The spliced leader (SL) RNA is transcribed independently and contains two domains, a 39-nucleotide exon and an intron. The 5' end of the SL-RNA contains a specialized "cap 4" structure in which a ^{7}mG is attached to the first nucleotide and the first four nucleotides as well as the sixth nucleotide are methylated. Thus *trans*-splicing of the 39-nucleotide exon allows all mRNAs to acquire the cap 4 structure, a feature that may be important in transport, stability and translation (6). The fundamental mechanism of *trans*-splicing is believed to be similar to that of *cis*-splicing and requires the participation of several small nuclear RNAs (snRNAs). The SL RNP ribonucleoprotein particle of which SL RNA is a

component and the U2, U4 and U6 snRNPs have been implicated in the *trans*-splicing reaction (7). However, the details of this process have yet to be worked out.

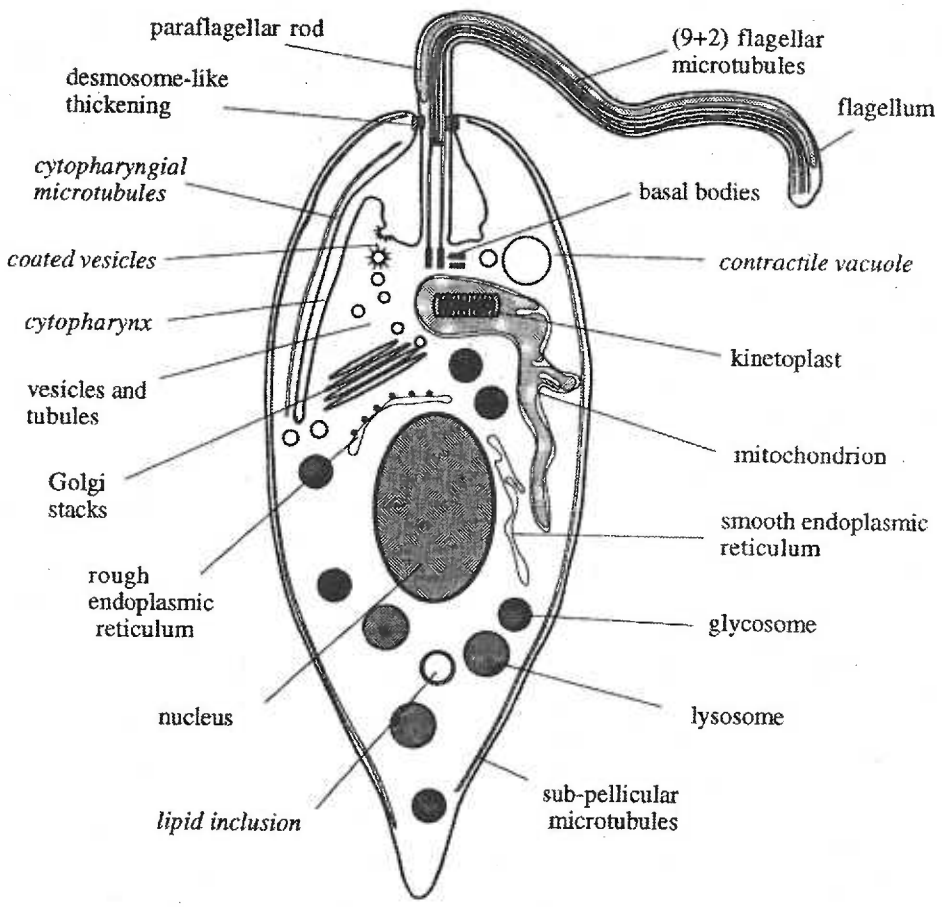
2. *Leishmania* cell biology

Leishmania possess all the classical membrane bound organelles of eukaryotic cells such as the nucleus, mitochondrion, endoplasmic reticulum, Golgi apparatus and secretory vesicles (Figure 1-1). *Leishmania* promastigotes (the insect stage of the parasite) have a spindle shaped body with a single flagellum whereas amastigotes (the mammalian stage) are roughly spherical with only a flagellar remnant. A corset of closely spaced sub-pellicular microtubules located beneath and attached to the plasma membrane of the cell body maintains the shape of these parasites (8). In addition to maintaining the form of the parasite, these sub-pellicular microtubules may act as a barrier against vesicular fusion (9, 10). At the anterior end of the cell the flagellum arises from a specialized invagination of the plasma membrane called the flagellar pocket. The flagellar pocket membrane lacks an underlying network of microtubules and consequently is the only part of the cell surface that supports endocytosis and exocytosis (10). Thus all proteins that are being endocytosed or exocytosed must pass through the flagellar pocket. At the opening of the flagellar pocket, hemi- desmosomal junctions are present between the membranes of the cell body and the flagellum. However, these junctions do not present a continuous barrier and large molecules like ferritin and antibodies can move in and out of the pocket (9).

The surface of promastigotes is coated by a number of glycosylphosphatidyl inositol (GPI)-anchored glycoproteins, GPI-anchored lipophosphoglycan (LPG) and a

Figure 1-1. General Features of a *Leishmania* Promastigote.

Taken from Clayton, C., Häusler, T. & Blattner, J. 1995. Microbiol. Rev. 59, 325-344.


paraflagellar rod
desmosome-like thickening
cytopharyngial microtubules
coated vesicles
cytopharynx
vesicles and tubules
Golgi stacks
rough endoplasmic reticulum
nucleus
lipid inclusion
(9+2) flagellar microtubules
flagellum
basal bodies
contractile vacuole
kinetoplast
mitochondrion
smooth endoplasmic reticulum
glycosome
lysosome
sub-pellicular microtubules

family of free GPIs termed glycoinositol phospholipids (GIPLs) (11). The zinc metaloprotease gp63 and lipophosphoglycan LPG are the most abundant GPI-anchored molecules on the surface of promastigotes. LPG forms a dense glycocalyx coat on the surface that is essential for the survival of the parasite in both the sandfly and the mammalian host (11). The GPI anchor precursors are assembled in the endoplasmic reticulum and then transferred to nascent proteins containing the appropriate C-terminal GPI signal sequence (11). Following their synthesis in the endoplasmic reticulum, these membrane proteins and glycolipids pass through the Golgi apparatus where they may be further modified (10, 12, 13). Organelles involved in the transport of membrane-bound molecules are clustered at the anterior end of the cell (14). Membrane proteins traffic through the *trans* Golgi network composed of tubovesicular structures and are inserted by vesicular fusion into the membrane of the flagellar pocket. From there, GPI-anchored and other membrane proteins can move by lateral diffusion to the surface of both the cell body and the flagellum (10). The flagellar pocket membrane may also serve as a station for sorting proteins destined for the flagellar membrane from those for the pellicular plasma membrane (15).

Kinetoplastids possess organelles called glycosomes that are evolutionarily related to peroxisomes and glyoxysomes of higher eukaryotes. Like peroxisomes, glycosomes contain enzymes of fatty acid oxidation and pyrimidine synthesis. Unlike peroxisomes, however, glycosomes also contain the first seven enzymes of the glycolytic pathway and two enzymes of glycerol metabolism (9).

3. Life cycle

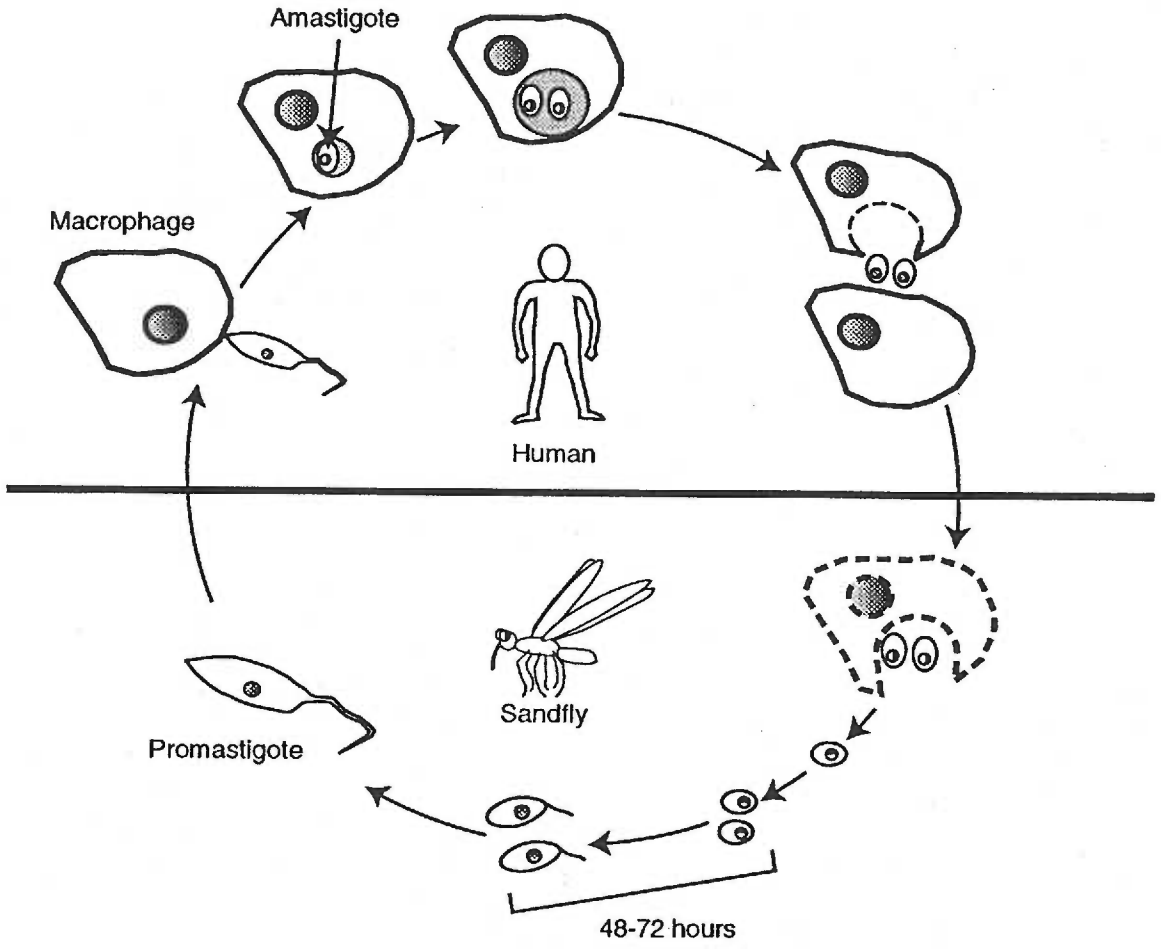
Leishmania are digenetic organisms that cycle between insect and mammalian hosts during the course of their life cycle (Figure 1-2). All *Leishmania* species are transmitted to mammalian hosts by sandflies belonging to the genus *Phlebotomus* (in the Old World) and *Lutzomyia* (in the New World) (16). In the sandfly the parasites exist as extracellular, flagellated and motile promastigotes within the lumen of the gut. Upon entry into the mammalian host, however, they transform into aflagellated and nonmotile amastigotes that survive and replicate as obligate intracellular parasites within parasitophorous vacuoles of mononuclear phagocytes (16, 17). The parasite, in shuttling between these two hosts is exposed to extreme environmental changes. The pH within the sandfly gut is estimated to be > 8.5 and the temperature to be between 22°C and 28°C. Within the phagolysosome of the macrophage the parasite encounters a more acidic pH of 4.5 - 6.0 and temperatures close to 37°C (18). The virulence of *Leishmania* parasites stems in part from their ability to withstand and adapt to such dramatic changes in their environments.

3.1 Survival and development within the sandfly vector

When a sandfly takes a blood meal from an infected mammalian host, macrophages containing *Leishmania* amastigotes are introduced into the lumen of the sandfly midgut. The bloodmeal becomes enclosed in a sac-like peritrophic membrane (PM) secreted by the midgut epithelium, within which the amastigotes continue to go through a few cell divisions (17). However, the parasites soon sense the change in their environment and initiate a series of metabolic changes resulting in their transformation

Figure 1-2. *Leishmania* life cycle.

Leishmania exhibit a digenetic life cycle, alternating between an invertebrate (sand fly) and vertebrate (mammal) host.


Amastigote
Macrophage
Human
Sandfly
Promastigote
48-72 hours

into non-infectious, motile promastigotes (19). This process is highly regulated and accompanied by the expression of developmental stage-specific proteins (19). The development of parasites within the sandfly is complex and several discrete stages can be identified (19). Amastigotes transform into short, ellipsoid procyclic promastigotes that are about 6 – 8 μm long. These non-infectious procyclic promastigotes continue to divide within the PM but gradually transform into the more elongated nectomonad forms (15 – 20 μm long). This transformation is apparently dependent on cell density and is possibly triggered by the depletion of nutrients in the bloodmeal. After ~ 3 days the PM begins to breakdown, allowing the nectomonads to migrate to the anterior midgut where they attach to the epithelium by inserting their flagella between the microvilli (19). Anchoring to the midgut epithelium prevents parasite loss during the passage of the bloodmeal and allows the parasite to complete its development within the sand fly (20).

One of the major surface molecules on *Leishmania* promastigotes is the glycoconjugate lipophosphoglycan (LPG). LPG, a tripartite molecule composed of a phosphoglycan (PG) domain linked *via* a hexasaccharide glycan core to a 1-O-alkyl-2-lysophosphatidylinositol anchor, is expressed over the entire surface of the parasite including the flagellum (20). Recent studies by Sacks *et al.* provide compelling evidence that LPG plays a critical role in mediating the binding of the parasite to the midgut epithelium and thus preventing parasite loss during excretion of the bloodmeal (21). Although the PG moieties from all *Leishmania* species share a common backbone of repeating disaccharide units of PO_4-6Galβ(1>4)Manα1, they exhibit differences in

substitutions at position 3 of the Gal residues (20, 21). Interestingly, these polymorphisms in LPG structure determine the ability of particular species of *Leishmania* to successfully colonize different sandfly species (20). For example, the *L. major* LPG which contains several glycan side chains, binds strongly to the midgut of *Phlebotomus papatasi*, the natural host for *L. major*. *L. donovani*, on the other hand is transmitted by *P. argentipes* and *L. donovani* LPG which lacks any substituted glycan side chains binds to the midgut epithelium of *P.argentipes* but not to that of *P. paptasi* (20). The phosphoglycan moieties play another important role. Within the midgut the parasites must resist the action of proteolytic enzymes secreted during the digestion of the blood meal. In addition to LPG, the PG repeats are also associated with secreted proteins such as proteophosphoglycan (PPG) and secreted acid phosphatase (sAP). Furthermore, *Leishmania* promastigotes also secrete the PG repeats as a hydrophilic structure. These secreted PG-containing products protect the parasite from digestive enzymes released in their vicinity and thus play an essential role in parasite survival within the midgut (21).

About 5 days after the ingestion of the bloodmeal, the parasites differentiate into highly motile and infectious metacyclic promastigotes that colonize the anterior midgut and foregut of the sandfly lumen (19). The process of metacyclogenesis is complex and involves changes in gene expression and alterations at the cell surface (16). LPG undergoes structural changes leading to the thickening of the glycocalyx coat. In *L. major* there is a reduction in the number of galactose residues in the LPG side chains and an increase in the number of terminating arabinose residues (16, 17). These

structural changes during metacyclogenesis alter the ability of LPG to interact with the midgut epithelium and allow the promastigotes to migrate forward into the mouthparts (19). Moreover, the length of the LPG molecule increases dramatically due to the doubling of the number of repeating disaccharide units. This elongated LPG protects the parasite surface from the lytic activities of the mammalian complement system (22). The major surface zinc metaloprotease gp63 is also upregulated during metacyclogenesis. In certain species of *Leishmania*, metacyclogenesis is accompanied by the expression of a metacyclic-specific isoform of gp63 (17).

3.2 Survival within the mammalian host

Metacyclic promastigotes are introduced into the mammalian host when an infected sandfly takes its next blood meal. The parasites are deposited into the pool of blood generated by the bite of the sandfly, where they must first survive the lytic activity of the host complement system. Survival within the host thus depends on the ability of the parasites to become intracellular. The entry of parasites into mononuclear phagocytes is a receptor mediated process involving mainly complement receptor types I and III (CR1 and CR3) on the surface of the macrophages and the third component of complement on the parasite (C3b and iC3b) (16, 17, 23). The available literature on the mechanism of complement activation by the parasite is confusing. Early studies suggested that promastigotes activated the alternative pathway of complement (16). However, whether the classical or alternative pathway is activated appears to be *Leishmania* species specific and depends at least in part on the structure of the LPG. Metacyclic promastigotes of *L. major* activate the classical pathway leading

to the deposition of C3b (complement component 3) on the surface mainly *via* an ester linkage with LPG, whereas *L. donovani* and *L. mexicana* apparently activate the alternative pathway (16). Recent studies however, suggest that *L. donovani* promastigotes activate only the classical complement pathway in humans (24). Regardless of the mechanism, activation of complement leads to the opsonization of the parasite with C3b, the ligand for CR1 on the macrophage surface. Although both procyclic and metacyclic promastigotes bind C3b, metacyclic organisms are more resistant to lysis by complement. This is in part due to the thick LPG coat on the surface of the latter which prevents the membrane attack complex from reaching the parasite surface (17, 22). Gp63, the surface metaloprotease, which is upregulated during metacyclogenesis also contributes to parasite survival. By enhancing the rate of cleavage of C3b to the inactive form iC3b, gp63 reduces the amount of terminal complement components on the parasite surface and protects the parasite from lysis (25). Moreover, as the iC3b generated serves as the ligand for the macrophage receptor CR3, gp63 enhances binding of the opsonized parasites to macrophages (25). Studies by Rosenthal *et al.* have shown that although metacyclic *L. major* promastigotes bind to both CR1 and CR3 on human macrophages, CR3 is the only receptor used for internalization. Blocking CR1 did not alter phagocytosis of complement opsonized promastigotes whereas blocking CR3 significantly reduced phagocytosis (26). Interestingly, CR1 behaves as a cofactor for factor I-mediated cleavage of C3b to iC3b and may actually facilitate the generation of ligands for CR3 (26).

3.3 Phagocytosis

Phagocytosis of microorganisms by the macrophage usually triggers the microbicidal oxidative burst. Although promastigotes possess a superoxide dismutase that can convert the superoxide anion to hydrogen peroxide, they do not have adequate amounts of enzymes like catalase and glutathione peroxidase to scavenge all the toxic oxygen metabolites that are produced during the burst (27). However, *Leishmania* promastigotes mainly engage complement receptors CR1 and CR3 on the macrophage, receptors that do not trigger the respiratory burst (22). The macrophage NADPH oxidase that initiates the phagocytic burst has to be activated by phosphorylation of one or more of its subunits by protein kinase C (27). *Leishmania* LPG can inhibit protein kinase C and therefore inhibit or at least minimize the phagocytic burst (17, 22, 23, 27). Moreover, LPG is also capable of scavenging oxygen free radicals generated during the oxidative burst. Macrophages express iNOS (cytokine-inducible NO synthase) in response to stimulation by a range of cytokines including interferon-γ (IFN-γ) and tumor necrosis factor α (TNF α). iNOS catalyzes the synthesis of NO from L-arginine and molecular oxygen and NO is a potent microbicidal agent involved in the killing of a range of microorganisms including *L. major*. LPG can regulate the activity of iNOS and therefore profoundly affect the survival of *Leishmania* within macrophages (28). Gp63 also aids in parasite survival by inhibiting the oxidative burst and its protease activity has been implicated in protecting the parasite from lysosomal cytolysis. Following endocytosis, phagosomes containing *Leishmania* parasites fuse with lysosomes and/or late endosomes to form

parasitophorous vacuoles (PVs) (29). PVs have an acidic luminal pH of 5 and contain several active acidic hydrolases. The PV membrane contains lysosomal proteins like LAMP-1 and LAMP-2 as well as proteins of the late endosomal compartment such as the cation-independent mannose-6-P receptor. Thus the PV is really a mixed organelle with both late endosomal and lysosomal characteristics (29).

Membrane proteins of amastigotes are more resistant to proteolysis than promastigote membrane proteins (17). In this light it is interesting that the promastigote LPG has the ability to transiently inhibit phagosome-endosome fusion, thereby allowing promastigotes to transform into amastigotes, forms that are better adapted to conditions within the PV (30). The transformation into amastigotes is accompanied by the reduction or loss of LPG expression and concomitant phagosome-endosome fusion (30).

3.4 Transformation into amastigotes

The temperature shift to 37°C and change in pH to 5 trigger the transformation of the promastigotes into amastigotes, a process that takes 2-5 days depending on the *Leishmania* species (29). Although the details of how this transition occurs remain to be worked out, it clearly involves changes in the expression of several genes. The amastigote metabolism is adapted to an acidic pH. Thus while promastigotes of *L. mexicana* express gp63 isoforms that have optimal proteolytic activities at extracellular pH values of ~ 7, the gp63 in amastigotes functions best at pH values of ~ 5.5 (18). Similarly, the proton motive force dependent transport of proline (an important energy source in the sandfly stage) occurs optimally at pH 7.5 in promastigotes but at pH 5.5

in amastigotes. To survive such drastic changes in external pH during their life cycle, *Leishmania* must maintain constant internal pH values. Indeed, promastigotes and amastigotes of *L. donovani* maintain constant intracellular pH values of 6.8-7.4 at extracellular pHs ranging from 4.5 to 7.5 (18). The proton translocating ATPase located on the parasite plasma membrane has been implicated both in creating a transmembrane proton electrochemical gradient and in regulating internal pH (18, 31). Parasites must also adapt to a rapid exposure to higher temperatures within the mammalian host. Interestingly, *Leishmania* species differ in their abilities to withstand temperature stress and this determines the tropism of the species (18). Thus *L. major* and *L. mexicana* multiply better in macrophages at 35°C than at 37°C and are therefore restricted to skin lesions where the temperature is about 35°C. On the other hand, *L. donovani* multiplies equally well at 35°C and 37°C and establishes lesions in visceral organs where the temperature is 37°C. The increase in temperature induces the expression of genes encoding several heat shock proteins including hsp70 and hsp83 (18). In addition to heat shock proteins several other amastigote specific proteins have been identified. These include the A2 protein from *L. donovani* (32) and members of multigene families such as the parasite surface antigen 2 (PSA 2) and gp63 of *L. major* that are selectively expressed in amastigotes (17). These amastigote specific proteins presumably aid the parasite in surviving the hostile conditions within the macrophage but the mechanism of their actions is not understood. The promastigote to amastigote transformation is also accompanied by major metabolic changes. There is a dramatic reduction in the rate of respiration and glucose catabolism (17,23). Concomitantly fatty

acids become the predominant source of energy for amastigotes (23). There are also changes at the cell surface. Both LPG and gp63 are downregulated in amastigotes (17). *L. major* amastigotes possess ~ 1000-fold less LPG than promastigotes whereas *L. donovani* and *L. mexicana* amastigotes do not express any LPG at all. However, amastigotes have an increased ratio of glycolipids to proteins at the cell surface and are particularly rich in glycoinositol phospholipids (GIPLs) (17). These GIPLs form a dense coat on the amastigote surface and apparently mediate the binding of amastigotes to macrophages (33). GIPLs also promote amastigote survival by inhibiting microbicidal activities such as NO production (17) and protect the parasites against the acidic medium and hydrolases within the PV (29). Interestingly, amastigotes have the ability to internalize and degrade major histocompatability complex (MHC) class II molecules that reach the PV in organelles called megasomes that are rich in cysteine proteinases (29). In doing so, the parasites reduce the number of parasite-MHC class II molecule complexes on the surface of the macrophage and escape recognition by the host immune system (29).

It is commonly accepted that after several cycles of replication, the macrophages burst releasing the amastigotes, which can then re-infect other host cells. However, it is also possible that the release of amastigotes occurs by the fusion of the PV with the macrophage plasma membrane (29). For the *Leishmania* infection to persist the released amastigotes must re-infect new host cells. Since amastigotes lack abundant LPG and gp63 molecules at the surface, they must use different strategies to gain entry into macrophages. However, much less is known about amastigote entry into

macrophages than is known about promastigotes. Amastigotes bind non-specifically to several different cell types (33). Lesion derived amastigotes are opsonized with immunoglobulins and it is believed that internalization via the Fc receptors is the major route of entry into macrophages and granulocytes (33). The non-specific adhesion mechanism could however be important for other cell types that take up parasites such as neutrophils and fibroblasts (33).

Recovery from *Leishmania* infection depends upon the production of interleukin-12 by activated macrophages (22). IL-12 drives the proliferation of CD4+ Th 1 cells and induces interferon-γ production by T cells. IFN-γ induces macrophages to produce iNOS and NO leading to parasite killing.

4. Leishmaniasis

Leishmaniasis is endemic in 88 countries on 5 continents with about 350 million individuals at risk (34). According to World Health Organization estimates, 12 million individuals worldwide are affected by leishmaniasis and there are 1.5-2 million new cases every year. Since 1993, the number of cases of leishmaniasis has increased sharply in areas already endemic for the disease. Wars, extensive environmental degradation and rural-urban migration are some of the factors that are believed to have contributed to the spread of the disease. In southwestern Europe the coexistence of HIV and leishmaniasis has emerged as a serious problem. Of the 1700 cases of co-infection reported to the WHO up to the year 1998, from 33 countries worldwide, 1440 cases were from southwestern Europe (34).

There are several species of *Leishmania*, but those that are pathogenic to humans can be grouped into three species complexes, the *Leishmania (L.) donovani* complex, the *L. mexicana* complex and the *L. braziliensis* complex. Together these species produce a spectrum of diseases including visceral, cutaneous and mucosal leishmaniasis (35).

4.1 Visceral leishmaniasis

Members of the *L. donovani* group are responsible for visceral leishmaniasis, the most severe form of the disease. The disease is caused primarily by *L. donovani* in the Indian subcontinent and Africa, *L. infantum* in Mediterranean regions and *L. chagasi* in the New World (36). In most individuals, infections are asymptomatic and parasites are eliminated by effective cell-mediated immune responses. However, in persons with the disease parasites are disseminated to macrophages throughout the reticulo-endothelial system causing hepatomegaly and massive splenomegaly (35). Symptomatic visceral leishmaniasis is commonly fatal if untreated.

4.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis is caused by members of the *L. mexicana complex* (*L. mexicana mexicana, L. mexicana amazonensis and L. mexicana venezuelensis*) and the *L. braziliensis* complex in the New World and by *L. tropica* and *L. major* in the Old World (36). Cutaneous leishmaniasis is characterized by the development of a chronic skin lesion at the site of parasite inoculation. Over a period of time, the lesions ulcerate and eventually heal spontaneously. However, in infections by *L. mexicana amazonensis* and *L. mexicana pinafoi,* the parasites that initially multiply at the site of inoculation can eventually spread to macrophages throughout the skin forming metastatic lesions and

producing an extremely disfiguring condition called diffuse cutaneous leishmaniasis (35).

4.3 Mucosal leishmaniasis

In a small proportion of individuals infected with members of the *L. braziliensis* group, infections may spread to the mucous membranes of the nose, mouth and pharynx several years after their cutaneous lesions have resolved. These destructive mucosal lesions do not heal spontaneously (35).

5. Chemotherapy

Pentavalent antimonials have been the frontline drugs in the treatment of leishmaniasis since 1912 (36). Sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) are used today to treat all forms of leishmaniasis. Although the mechanism of action of these antimonials is not known, several studies indicate that they may act by inhibiting the initial steps in glycolysis as well as certain enzymes of the Kreb's cycle (37). These drugs are given at high doses (20 mg/kg per day) and require prolonged treatment schedules (20 –30 days). At these dosage regimens these drugs produce several toxic side effects including chemical and symptomatic pancreatitis, musculoskeletal pain and occasionally cardiotoxicity (36). The emergence of clinical isolates that are resistant to antimonials is another significant problem (36). Pentamidine has been used to treat visceral leishmaniasis in regions where resistance to antimony prevents its use (36). However, the high-dose and long-course regimen of pentamidine used for visceral leishmaniasis, makes it even more toxic than pentavalent antimony. Amphotericin B formulations are now being used increasingly in anti-

leishmanial chemotherapy to treat antimony-resistant infections. Less toxic, lipid associated amphotericin B (deoxycholate) formulations such as liposomal amphotericin B (L-AmB [AmBisome]), amphotericin B colloidal dispersion (ABCD [Amphocil]) and amphotericin B lipid complex (ABLC [Abelcet]) are now available for clinical use. Although these formulations can be administered at lower doses and are less toxic, they are extremely expensive (36). Clearly, less toxic and more efficacious drugs are urgently needed.

Biochemical pathways that are distinct between the parasite and its host are ideal targets for rational drug design. Because purine metabolism in *Leishmania* is significantly different from that in humans, it offers several opportunities for chemotherapeutic intervention. Purine metabolism is discussed in the next section.

6. Purine and pyrimidine metabolism

Purine and pyrimidine nucleotides are involved in a number of biochemical processes. They are the fundamental units of the nucleic acids DNA and RNA, and serve as cellular energy sources in the forms of ATP and GTP. They are also components of coenzymes such as NAD^+, FAD and coenzyme A.

6.1 Purine biosynthetic pathways in mammalian cells

Most mammalian cells possess two pathways for the synthesis of purine nucleotides; a *de novo* pathway that leads to the synthesis of inosine monophosphate (IMP) from non-purine precursors such as amino acids and carbon dioxide and a salvage pathway that reutilizes preformed purines (38). The *de novo* pathway consists of eleven enzymatic reactions that assemble the purine ring on a ribose-5-phosphate

backbone, leading to the synthesis of IMP, the precursor for both adenine (AMP) and guanine (GMP) nucleotides (39). This pathway is energetically expensive, requiring 6 mols of ATP per mol of purine nucleotide synthesized (39). Most mammalian cells also possess a purine salvage pathway that reutilizes preformed purine bases and nucleosides released during the degradation of nucleic acids (38, 40). These pathways are especially important in cells such as enterocytes and bone marrow cells that lack *de novo* biosynthetic pathways and depend solely on salvage for their purine requirements. Salvage pathways in mammals are similar to those in *Leishmania* described in detail below. In humans purine nucleotides that are not salvaged are catabolized and excreted as uric acid, thus allowing cells to maintain constant internal levels of purine nucleotides in the face of continuous *de novo* synthesis (39). The purine catabolic pathway begins with the dephosphorylation of purine nucleoside monophosphates to their corresponding nucleosides by specific 5′ nucleotidases or non-specific phosphatases. These nucleosides are cleaved into nucleobases by purine nucleoside phosphorylases, and oxidized by xanthine oxidase to uric acid (39).

6.2 Purine metabolism in *Leishmania*

Purine metabolism in *Leishmania* differs significantly from that in humans in several ways. First, *Leishmania* and all other parasitic protozoa lack the *de novo* pathway for purine nucleotide synthesis, and depend entirely on salvage to meet their purine requirements. These parasites have acquired a distinct set of purine salvage enzymes that enable them to scavenge host purines efficiently. Some of the enzymes in this pathway are unique to *Leishmania* and do not have counterparts in mammalian cells.

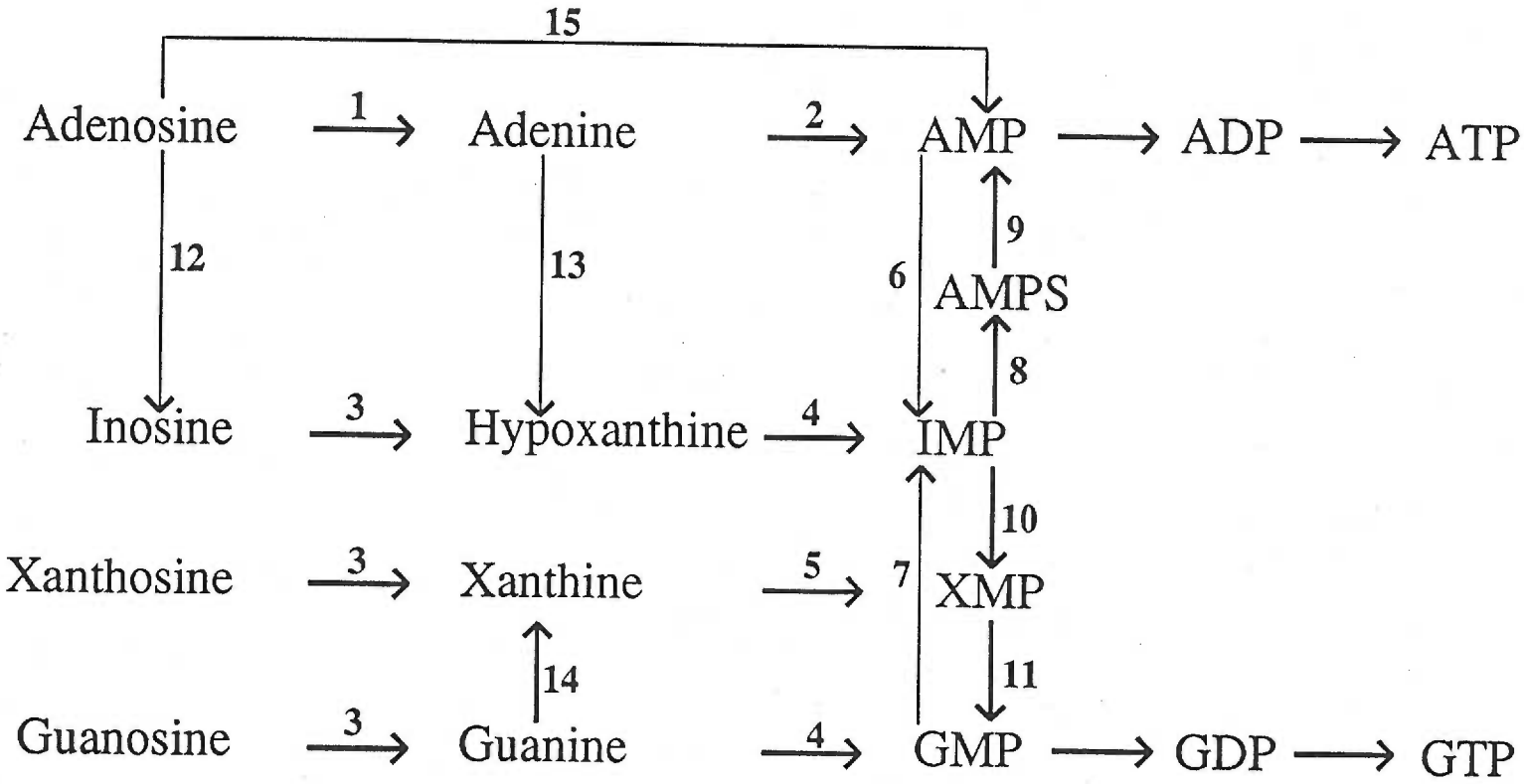
Finally, since *Leishmania* do not have xanthine oxidase, there is no purine catabolism in these parasites (38).

6.2.1 Purine salvage in *Leishmania*

Leishmania parasites salvage both purine bases and nucleosides (Figure 1-3). Purine bases are phosphoribosylated by specific purine phosphoribosyltransferases (PRTs) to yield nucleoside-5′-monophosphates and PPi. Nucleosides can be first cleaved into purine bases by purine nucleoside hydrolases or phosphorylases and then utilized in the PRT reaction, or directly phosphorylated to nucleotides by specific nucleoside kinases (38).

Figure 1-3. Purine Salvage and Interconversion pathways in *Leishmania*.

Enzymes listed are as follows: 1. Nucleoside phosphorylase; 2. APRT; 3. Nucleoside hydrolase; 4. HGPRT; 5. XPRT; 6. AMP deaminase; 7. GMP reductase; 8. Adenylosuccinate synthetase; 9. Adenylosuccinate lyase; 10. IMP dehydrogenase; 11. GMP synthetase; 12. Adenosine deaminase (amastigotes); 13. Adenine deaminase (promastigotes); 14. Guanine deaminase; 15. Adenosine kinase.


15
Adenosine
1
Adenine
2
AMP
ADP
ATP
12
13
9
6
AMPS
8
Inosine
3
Hypoxanthine
4
IMP
10
Xanthosine
3
Xanthine
5
7
XMP
14
11
Guanosine
3
Guanine
4
GMP
GDP
GTP

Central to the *Leishmania* salvage pathway, are the reactions catalyzed by the purine phosphoribosyl transferases. These enzymes catalyze the divalent cation dependent reversible reaction between free purine bases and PRPP to yield the purine nucleoside monophosphate and PPi. Catalysis is believed to proceed by an SN-1 type reaction mechanism with the formation of a positively charged oxycarbenium ion transition state and accompanied by an inversion of stereochemistry at the C1 position of the ribose moiety (41). There are three distinct PRTs in *L. donovani* with different substrate specificities (38). Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is specific for hypoxanthine and guanine while adenine phosphoribosyl transferase (APRT) and xanthine phosphoribosyl transferase (XPRT) phosphoribosylate adenine and xanthine respectively. The *Leishmania* XPRT also accepts hypoxanthine as substrate. Mammalian cells lack XPRT and are thus incapable of utilizing xanthine as a purine source (38). Other parasitic protozoa such as *Plasmodium falciparum, Toxoplasma gondii* and *Tritrichomonas fetus* contain an HGXPRT that recognizes xanthine in addition to hypoxanthine and guanine, albeit less efficiently (38). *L. donovani* HGPRT on the other hand has a strict specificity for hypoxanthine and guanine and will not accept xanthine as substrate (42). Consequently, *Leishmania* parasites with a deletion of the XPRT locus are incapable of growth in media containing xanthine as the sole purine source (43). However, the *Leishmania* HGPRT is capable of accepting certain pyrazolopyrimidine analogs of hypoxanthine as substrates as discussed later in this chapter.

L. donovani promastigotes possess extremely active adenine and guanine deaminases that rapidly deaminate adenine and guanine to hypoxanthine and xanthine respectively (44). Thus most purine bases in promastigotes are ultimately salvaged by the HGPRT and XPRT reactions. However, APRT appears to play a more important role in *L. donovani* amastigotes that lack adenine deaminase and thus depend entirely on APRT to salvage adenine (38).

The genes for the *L. donovani* HGPRT (45), APRT (46) and XPRT (43) have been cloned, and the proteins purified and characterized. In addition, the crystal structure of APRT from *L. donovani* has been solved (47). Immunofluorescence studies have localized the *Leishmania* HGPRT to the glycosome, an organelle unique to trypanosomatids where enzymes of the glycolytic pathway and other fuel metabolizing enzymes are located (48). Preliminary cell fractionation studies suggest that XPRT may also be a glycosomal enzyme (43). In contrast, the *Leishmania* APRT does not appear to be glycosomal.

The salvage of nucleosides begins with their cleavage to nucleobases by nucleoside hydrolases or phosphorylases. Three nucleoside hydrolases with different catalytic properties have been identified in *L. donovani* promastigotes (49). One of these is highly specific for purine 2′-deoxyribonucleosides and will not cleave purine or pyrimidine ribonucleosides. The second, a purine ribonucleoside hydrolase is specific for inosine and guanosine, while the third is a broad specificity nucleosidase that hydrolyses both purine and pyrimidine nucleosides, although it cleaves uridine with the highest efficiency (49). Recently, Shi *et al.* reported the crystal structure of a broad

specificity nucleoside hydrolase from the related parasite, *L. major* (50). This enzyme differed from the *L. donovani* broad specificity enzyme in showing a strong preference for inosine rather than for uridine. No adenosine nucleosidase activity has been detected in *L. donovani*, but an adenosine phosphorylase was identified in *L. tropica* (49). In both promastigotes and amastigotes of *L. donovani*, adenosine is directly phosphorylated to AMP by adenosine kinase (38). However, kinases for inosine, guanosine and xanthosine have not been detected in *L. donovani*. Mammalian cells lack nucleoside hydrolases and their nucleosides are cleaved by phosphorylases instead (38), another significant difference between host and pathogen purine salvage pathways.

6.2.2 Purine Interconversion Pathways

In *Leishmania* as in most organisms, IMP can be converted to both AMP and GMP by alternative pathways. AMP is synthesized from IMP by a two step process. First, adenylosuccinate synthetase catalyzes the GTP dependent reaction between IMP and aspartate to form succino-AMP. Succino-AMP is then cleaved by succino-AMP lyase to AMP and fumarate (40, 51). IMP dehydrogenase and GMP synthetase catalyze sequential reactions in the synthesis of GMP from IMP and GMP reductase catalyzes the reduction of GMP to IMP (38). The enzymes HGPRT, adenylosuccinate synthetase, succino-AMP lyase and GMP reductase play important roles in the metabolism of pyrazolopyrimidines as discussed later in this chapter.

A surface membrane-bound 3′-nucleotidase/nuclease from *L. donovani* has been purified and characterized (52). This enzyme is capable of hydrolyzing both

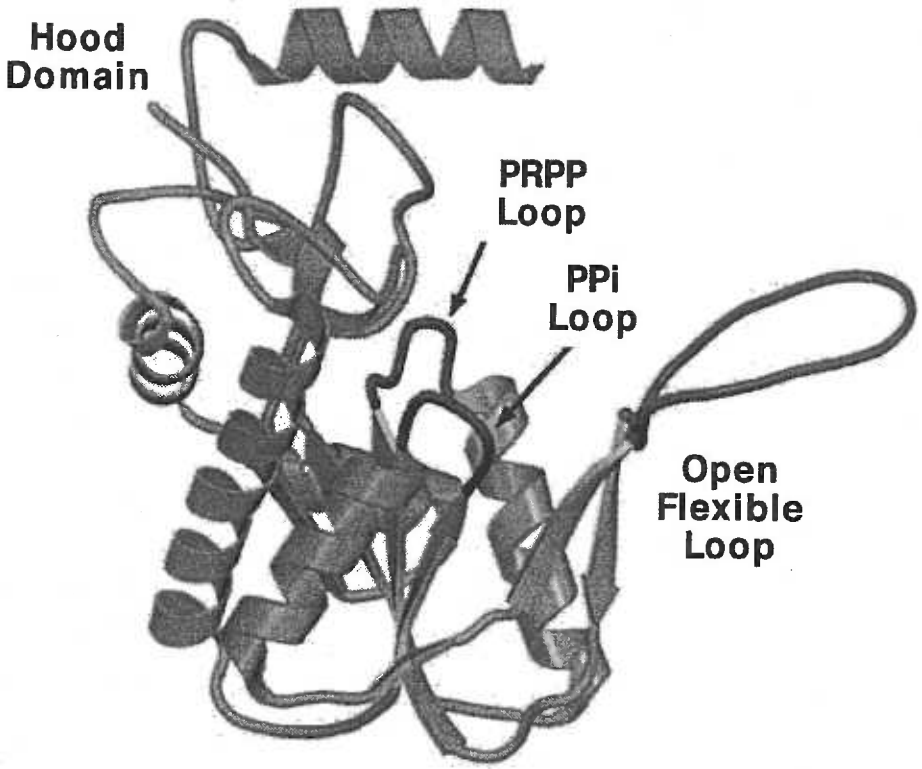
extracellular nucleotides and nucleic acids to nucleosides. It has been proposed that this enzyme plays an important role in purine salvage by generating purine nucleosides for transport (53).

6.3 Structural features of PRTs

In the past few years our understanding of the structure and function of the purine PRTs has advanced considerably. Crystal structures for a number of PRTs from diverse organisms are available (47, 54, 55). All these PRTs share certain structural features: a hood domain that binds the purine base and provides the nucleophile for the reaction, a core domain that provides the binding site for Mg-PRPP, and a flexible catalytic loop that closes over the active site during catalysis and shields the oxycarbenium ion transition state from the bulk solvent (Figure 1-4).

Figure 1-4: Structure of a Purine PRT.

Taken from Smith, J.L. 1999. *Nature Structural Biology* 6, 502-504.


Hood
Domain
PRPP
Loop
PPi
Loop
Open
Flexible
Loop

6.3.1 The Hood domain

The hood, which is structurally the most variable domain among the PRTs, provides the binding site for the nucleobase (41). Most of the interactions with the purine base involve residues in this domain and these interactions determine the substrate specificity of the PRT enzyme.

6.3.2 The Core Domain

The core domain is composed of a twisted β-sheet of 4-5 β strands flanked by 3-4 α helices (41). Three loops (PPi or loop 1, PRPP or loop 3 and a flexible catalytic loop, loop 2) located at the C-terminal edge of the central β-sheet provide the binding site for the Mg-PRPP. The PPi and PRPP loops form an extensive network of hydrogen bonds with the phosphate and pyrophosphate moieties of PRPP. A highly conserved sequence of 13 amino acids in the PRPP loop constitutes the PRPP binding motif and a pair of acidic residues within this motif forms hydrogen bonds with the 2′- and 3′-hydroxyls of the PRPP ribose (47, 54). These interactions help to position the PRPP in the binding pocket.

Another conserved acidic residue Asp 137 in the PRPP loops of HGPRTs forms a hydrogen bond with position N7 of the purine base and is believed to function as a general base during catalysis (54, 56). All three acidic residues are conserved in the *L. donovani* APRT and are likely to play similar roles (47).

Metal ions are absolutely essential for catalysis. APRTs have a single Mg^{2+} ion that coordinates with the 2′- and 3′-hydroxyls of the PRPP ribose, the β phosphate of

PPi and several water molecules (47). These interactions serve to position the PRPP in the active site and also to stabilize the negative charges on the PRPP oxygens. Both the human and *Trypanosoma cruzi* HGPRTs contain a second Mg^{2+} ion that in addition to coordinating with the pyrophosphate oxygens and several water molecules interacts directly with a residue located in the hood domain (Asp 193) (54, 55). Furthermore, one of the Mg^{2+} coordinated water molecules also forms a hydrogen bond with the purine position N3 of the hypoxanthine analog in the *T. cruzi* HGPRT structure (55). By interacting directly with the PRPP pyrophosphate and indirectly with the nucleobase, this Mg^{2+} ion plays a critical role in positioning both substrates for the nucleophilic attack at the C1 position of the ribose. In addition, the electron withdrawing tendency of the Mg^{2+} ions presumably aids in catalysis by activating the pyrophosphate leaving group.

6.3.3 The Catalytic Loop

Once both substrates are bound to the active site, the catalytic loop (loop 2) undergoes a large conformational change to cover the active site and shield it from the bulk solvent (41). However, in addition to its protective role, the flexible loop also appears to be important for binding PRPP and stabilizing the transition state. The main chain atoms of the conserved Ser 103-Tyr 104 dipeptide within this loop form hydrogen bonds with the pyrophosphate oxygens, while the side chain hydroxyl group of Tyr 104 interacts with the 5′ phosphate moiety (41). S103A and T104V mutations in the *L. donovani* HGPRT drastically reduce the turnover rate of the enzyme, thus underscoring the importance of this dipeptide in catalysis (57). A similar role has

been assigned to residues Glu120 and Tyr 121 in the catalytic loop of the *L. donovani* APRT (47).

Based on these structural and biochemical studies the following series of events is believed to occur during catalysis. The substrates bind and the flexible loop closes over the active site. Interactions between residues of the flexible loop and the hood bring the hood domain and the nucleophile down towards the core domain and allow the reaction to occur. Following these events, conformational changes cause the hood to move away and the flexible loop to open, thereby releasing the products (41, 58).

6.4 Pyrimidine metabolism in *Leishmania.*

6.4.1 The *de novo* pathway

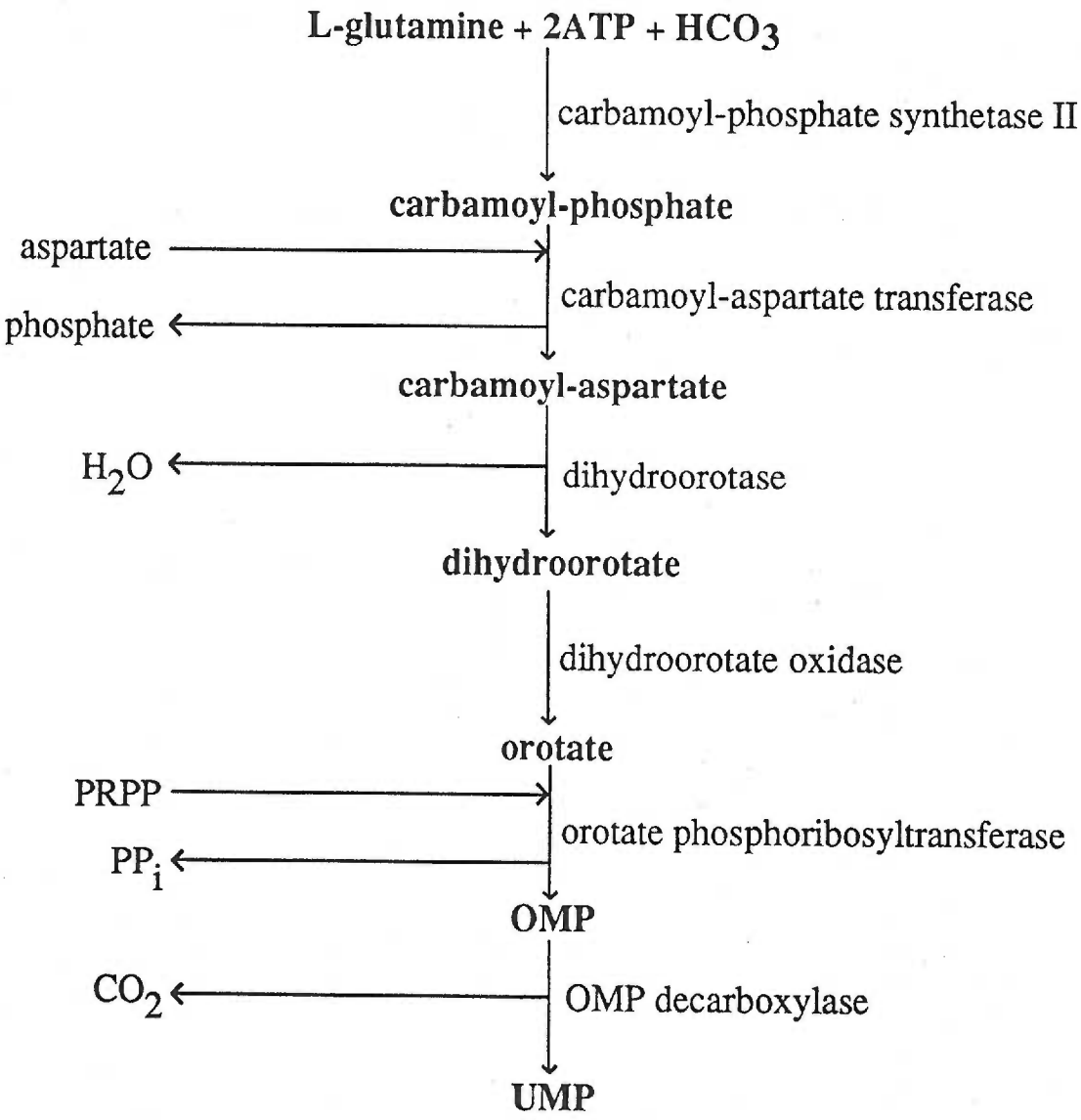
The ability of *Leishmania* promastigotes to grow in defined media in the absence of preformed pyrimidines suggested that these parasites were capable of synthesizing pyrimidines *de novo* (38). All the enzymes in the pyrimidine biosynthesis pathway have been identified in various *Leishmania* species (38, 59). This pathway includes six enzymatic reactions leading to the synthesis of UMP (Figure 1-5), which is the precursor for the other pyrimidine nucleotides (38,59).

Although the pyrimidine biosynthesis pathway in *Leishmania* is very similar to that in mammals, there are some differences. In mammals the first three reactions are catalyzed by a single multifunctional protein containing carbamoyl synthetase II, aspartate transcarbamoylase and dihydroorotase activities, whereas in *Leishmania* these reactions are catalyzed by distinct enzymes with separable activities (60). Furthermore, unlike the aspartate transcarbamoylase from several bacterial systems, the *Leishmania*

enzyme does not display any cooperative kinetics and is not highly regulated (60). Orotate phosphoribosyl transferase and OMP decarboxylase, enzymes that catalyze the phosphoribosylation of orotate and its subsequent decarboxylation to UMP, are believed to be associated with the external surface of glycosomes in *Leishmania*. In mammals, a bifunctional enzyme located in the cytoplasm catalyzes these reactions (38).

Figure 1-5. The *de novo* pyrimidine biosynthesis pathway in *Leishmania*.

Adapted from Hammond, D.J. & Gutteridge, W.E. 1984. *Mol. Biochem. Parasitol.* 13, 243-261.


L-glutamine + 2ATP + HCO_3
carbamoyl-phosphate synthetase II
carbamoyl-phosphate
aspartate
carbamoyl-aspartate transferase
phosphate
carbamoyl-aspartate
H_2O
dihydroorotase
dihydroorotate
dihydroorotate oxidase
orotate
PRPP
orotate phosphoribosyltransferase
PP_i
OMP
CO_2
OMP decarboxylase
UMP

6.4.2 Pyrimidine Salvage and Interconversion

As *Leishmania* are capable of synthesizing pyrimidine nucleotides *de novo*, pyrimidine salvage is not critical for their survival. Consequently, these salvage pathways are less efficient than those for purine reutilization are. The *Leishmania* pyrimidine salvage pathway is shown in Figure 1-6. *L. donovani* promastigotes possess a uracil phosphoribosyltransferase (UPRT) that catalyzes the phosphoribosylation of uracil to UMP (59). Mammalian cells lack a separate UPRT activity. Both orotate and uracil are phosphoribosylated by OPRT in mammals (38, 59). *L. donovani* promastigotes appear to be incapable of salvaging thymine (38), but salvage both orotate and thymidine, albeit very poorly (44). Most of the metabolized orotate is converted to uracil, which presumably is utilized in the UPRT reaction. A small proportion of the orotate is converted to UTP and CTP, but the enzymes catalyzing these reactions have not been identified. Almost 99% of the metabolized thymidine is converted to thymine, and the remaining to dTMP, dTDP and dTTP. None of the thymidine is metabolized to uridine, cytidine or their bases (44).

The synthesis of dTMP from dUMP is catalyzed by an unusual bifunctional thymidylate synthase (TS)/dihydrofolate reductase (DHFR) enzyme in *Leishmania* and other protozoa (61). In mammals, TS and DHFR are distinct monofunctional enzymes. TS catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate to dTMP and dihydrofolate, and DHFR catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. A separate enzyme, serine transhydroxymethylase (STH) subsequently regenerates the 5,10-methylenetetrahydrofolate for continued

dTMP synthesis (61). The *Leishmania* DHFR-TS is a dimer of two identical subunits, each with an amino terminal DHFR domain connected to a carboxy terminal TS domain by a linker region.

One advantage of a bifunctional DHFR-TS is that the synthesis of both enzymes can be coordinately controlled, so that as much DHFR is produced as is required to reduce the dihydrofolate synthesized by TS. However, a more important advantage is that the bifunctional enzyme allows the substrate to be rapidly channeled from the active site of TS to that of DHFR (61). Indeed, several studies have shown that the rate of transfer of dihydrofolate from the TS active site to that of DHFR and its subsequent reduction is faster than its release to bulk solvent (61). The crystal structure of the *Leishmania major* DHFR-TS provides evidence for such a kinetic channeling of substrate (62). The surface charge distribution of DHFR-TS is unusual in that, there is a highly positive electrostatic potential between and around the folate binding sites, but largely negative potential on the rest of the enzyme surface. This observation suggests that an electrostatic mechanism might operate to rapidly channel the negatively charged dihydrofolate product from one active site to the other (62).

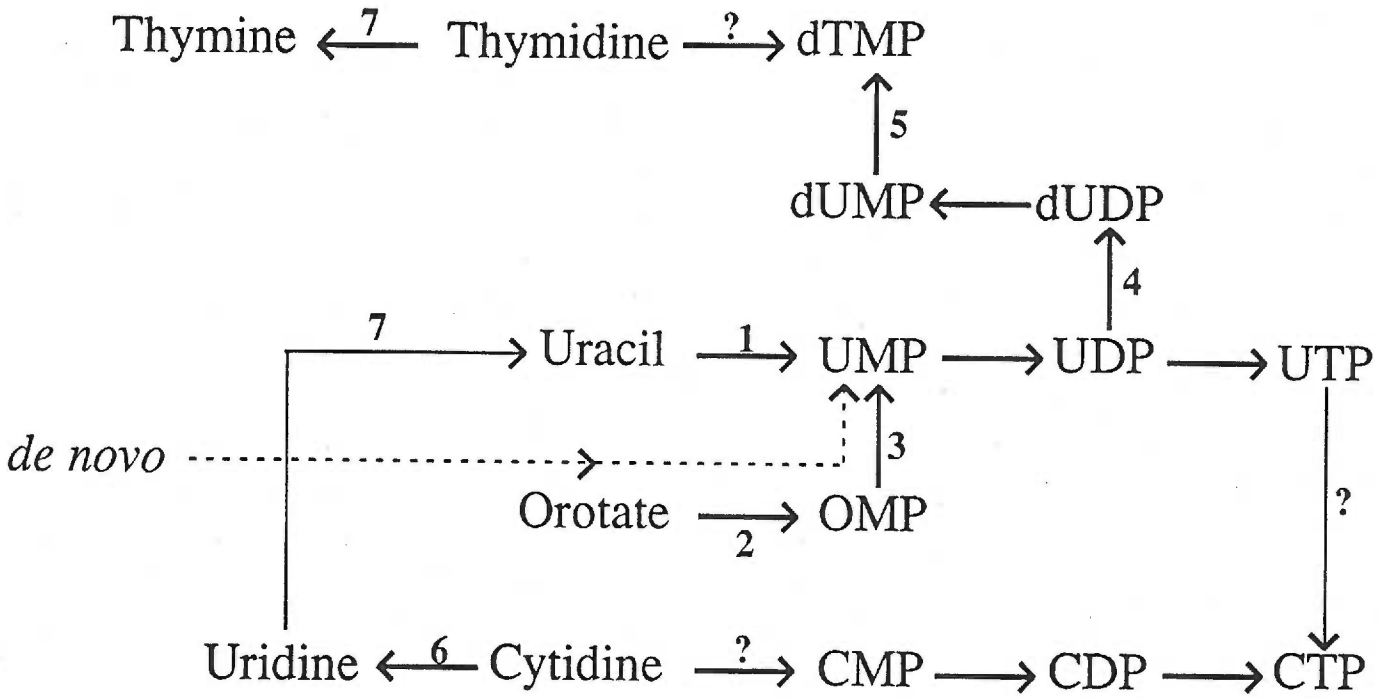
As described earlier, *Leishmania* promastigotes have a pyrimidine nucleosidase that cleaves both purine and pyrimidine nucleosides. This enzyme cleaves uridine most efficiently and appears to prefer an oxo substituent at position 6 of the pyrimidine ring (49).

The synthesis of purine and pyrimidine deoxyribonucleotides is catalyzed by ribonucleotide reductase (RNR) (38). The gene for the small subunit (M2) of RNR has

recently been cloned from hydroxyurea-resistant *Leishmania mexicana* mutants (63). Surprisingly, this subunit mainly localizes to the nucleus in *L. mexicana*, whereas the corresponding subunit in mammalian cells is cytoplasmic (64). Furthermore, while the synthesis of M2 is regulated and occurs only during the S phase of the cell cycle in mammalian cells, no cell cycle regulation of M2 synthesis was apparent in *L. mexicana* promastigotes (64).

Figure 1-6. Pyrimidine salvage and interconversion in *Leishmania*.

Enzymes listed are: 1. UPRT; 2. OPRT; 3. OMP decarboxylase; 4. Ribonucleotide reductase; 5. DHFR-TS; 6. Cytidine deaminase; 7. Nucleoside hydrolase.


Thymine
7
Thymidine
?
dTMP
5
dUMP
dUDP
4
7
Uracil
1
UMP
UDP
UTP
de novo
3
Orotate
2
OMP
?
Uridine
6
Cytidine
?
CMP
CDP
CTP

6.5 Metabolism of pyrazolopyrimidines in *Leishmania* and implications for chemotherapy

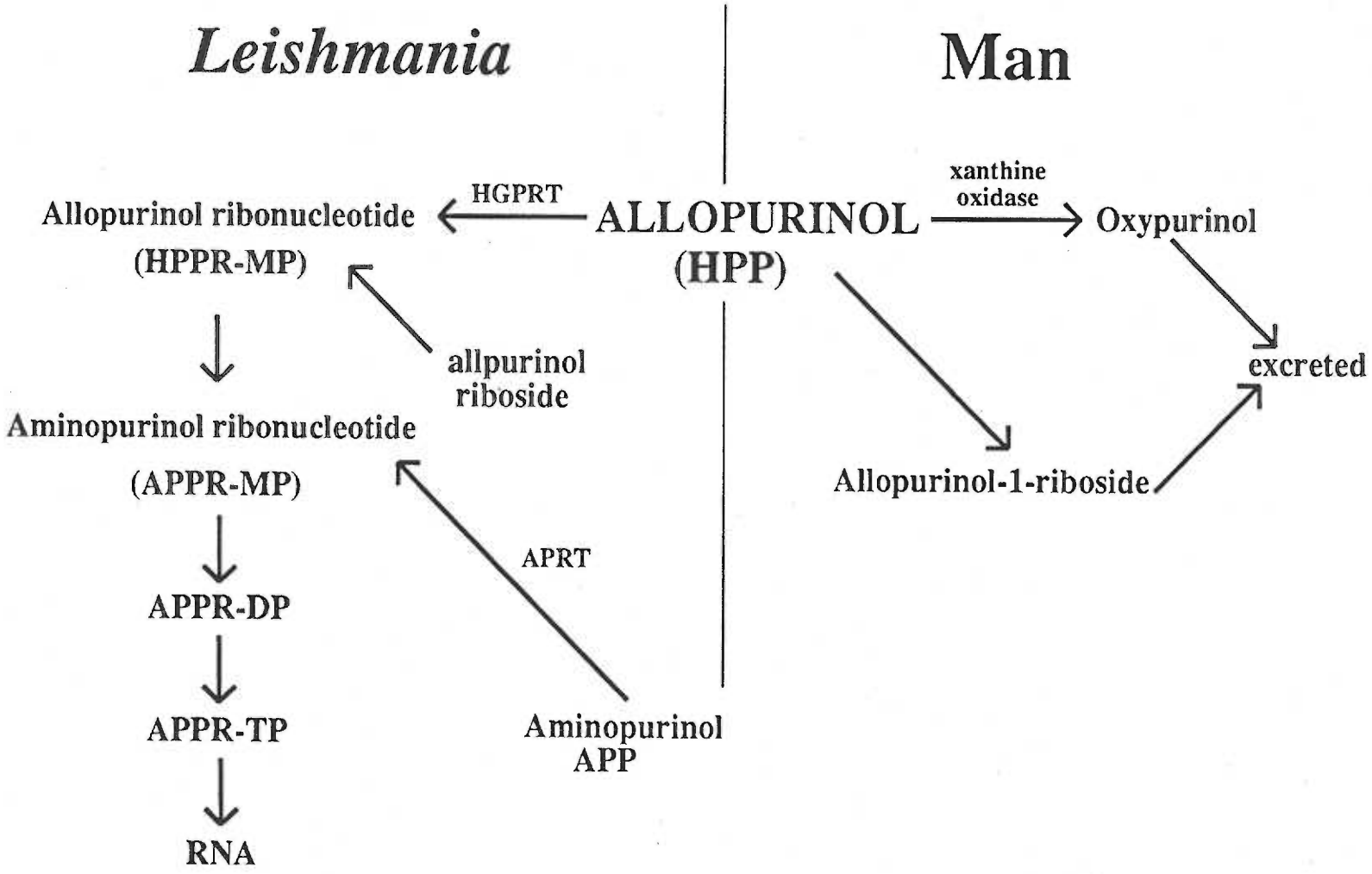
The pyrazolopyrimidine analogs of hypoxanthine, allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine; HPP), thiopurinol (4-thiopyrazolo[3,4-d]pyrimidine; TPP) and aminopurinol (4-aminopyrazolo[3,4-d]pyrimidine; APP) have antiprotozoal activities and inhibit the growth of *Leishmania* and other trypanosomatids (65). The metabolism of pyrazolopyrimidines in *Leishmania* is strikingly different from that in mammalian cells. This discrepancy is believed to result from differences in the substrate specificities of mainly two enzymes in the purine salvage pathway, HGPRT and adenylosuccinate synthetase (65). *Leishmania* HGPRT can phosphoribosylate both HPP and TPP to the nucleotides HPPR-MP and TPPR-MP respectively. Although these pyrazolopyrimidines are less efficient substrates than hypoxanthine and guanine, they are far better substrates of the leishmanial enzyme than of the mammalian counterpart (65, 66). As a result, these parasites accumulate large quantities of HPPR-MP to intracellular concentrations of 2-3 mM whereas mammalian cells do not. In humans, allopurinol is mainly oxidized by xanthine oxidase to oxypurinol, an inhibitor of xanthine oxidase that is used to treat hyperurecemia (66). Both TPPR-MP and HPPR-MP are potent inhibitors of GMP reductase and IMP dehydrogenase, enzymes that catalyze the interconversion of adenine and guanine nucleotides (67). At the concentrations present in *Leishmania,* these pyrazolopyrimidine nucleotides are capable of almost completely inhibiting

these enzymes and causing an imbalance in the ratio of adenine to guanine nucleotides. Significantly, these compounds have virtually no effect on the corresponding enzymes in mammals (67). Both IMP analogs, HPPR-MP and TPPR-MP also affect uracil metabolism in *Leishmania* and reduce intracellular UTP pools, although the mechanism is unclear (67). A second striking discrepancy in pyrazolopyrimidine metabolism between parasite and host, is the ability of the leishmanial but not the human adenylosuccinate synthetase to aminate HPPR-MP to succino-APPR-MP (51). Although this occurs at a very slow rate, the high intracellular concentrations of HPPR-MP allow it to compete effectively with IMP for the enzyme. Once formed, succino-APPR-MP is rapidly cleaved to APPR-MP by succinoAMP lyase, a relatively broad specificity enzyme in most organisms. Therefore, the selective amination of allopurinol ribonucleotide in *Leishmania* is due to the unique difference in the specificity of its adenylosuccinate synthetase (51).

APPR-MP, which is produced both by the amination of HPPR-MP and by the phosphoribosylation of APP by APRT, affects uracil and RNA metabolism. APPR-MP is metabolized to the triphosphate level and incorporated into RNA. This alteration in RNA structure apparently causes an increase in the breakdown of RNA with a concomitant reduction in protein synthesis (67). This ability of aminonucleotide analogs to interfere with RNA and protein metabolism is believed to be the major contributing factor to the anti-leishmanial properties of the pyrazolopyrimidines. Figure 1-7 depicts the differences in the metabolism of pyrazolopyrimidines between *Leishmania* and humans.

Figure 1-7. Pyrazolopyrimidine metabolism in *Leishmania* and humans.

Adapted from Meshnick, S.R. & Marr, J.J. 1982 in *Subcellular Biochemistry* **18** eds Avila, J.L. & Harris, J.R. (Plenum, NY) pp. 401-441.


Leishmania
Man
HGPRT
xanthine oxidase
Allopurinol ribonucleotide
(HPPR-MP)
ALLOPURINOL
(HPP)
Oxypurinol
allpurinol riboside
excreted
Aminopurinol ribonucleotide
(APPR-MP)
Allopurinol-1-riboside
APRT
APPR-DP
APPR-TP
RNA
Aminopurinol APP

As a result of these biochemical studies, considerable attention has now focused on pyrazolopyrimidines and allopurinol in particular as anti-leishmanial chemotherapeutic agents (68). In a clinical study, allopurinol was found to be very effective in the treatment of American cutaneous leishmaniasis (37). Allopurinol is particularly attractive because of its low toxicity to humans and because it is relatively inexpensive. There are also reports of the beneficial effects of allopurinol in the treatment of visceral leishmaniasis (68).

7. Nucleoside Transport

The salvage of purine nucleosides and nucleobases begins with their transport across the plasma membrane. Since these compounds are relatively hydrophilic, they cannot permeate the lipid bilayer of the plasma membrane and must be transported by specialized nucleoside and nucleobase transporters.

7.1 Nucleoside transport in mammalian cells

Most mammalian cells synthesize purine nucleotides *de novo*. However some cell types such as leukocytes, erythrocytes, intestinal epithelial cells and platelets lack *de novo* purine nucleotide biosynthetic pathways and depend on salvage to meet their purine requirements. Nucleoside transporters play an important role in the transmembrane permeation of nucleosides in these cell types (69). Moreover, in all cells excess nucleosides produced during the breakdown of nucleotides must be released. Thus the transmembrane movement of nucleosides occurs in almost all cell types.

In mammalian cells, nucleosides such as adenosine have a number of physiological and pharmacological effects. Adenosine acts as a local signaling molecule and affects lipolysis, neurotransmitter release and coronary vasodilation. These actions of adenosine are mediated via specific receptors (69, 70). Adenosine transporters play an important role in the pharmacological actions of adenosine by influencing the concentration of adenosine in the vicinity of these receptors. Thus inhibition of adenosine transporters greatly potentiates the actions of adenosine (69). Nucleoside transporters are also important in the transport of anti-cancer and antiviral drugs such as cytosine arabinoside (AraC), acyclovir, azidothymidine (AZT) and 5-fluorouracil.

In mammals two structurally unrelated protein families mediate nucleoside transport. The equilibrative nucleoside transporters (ENTs) are facilitative transporters that transport substrates down their concentration gradients, whereas the concentrative nucleoside transporters (CNTs) are secondary active transporters that utilize sodium gradients to drive the uphill transport of their nucleoside substrates (71).

7.1.1 Equilibrative Nucleoside transporters: Biochemical characterization

Equilibrative nucleoside transporters mediate the facilitated diffusion of nucleosides down their concentration gradients. These transporters are widely distributed in a variety of cell types and species and have been classified into two subtypes depending on their sensitivity to inhibition by the 6-thiopurine ribonucleoside nitrobenzylthioinosine (NBMPR) (72). The equilibrative sensitive (*es*)

transporters are potently inhibited by NBMPR with K_is of 0.1 to 10 nM. NBMPR competitively inhibits *es* transporter mediated nucleoside influx by binding tightly and reversibly to a site that is believed to overlap with the substrate binding site (69). These transporters can also be covalently radiolabeled with [^{3}H]NBMPR by exposure of the transporter-NBMPR complex to uv-light. Such photolabeling studies have helped in the quantitation of *es* type transporters in various mammalian cell types (69). In contrast, the equilibrative insensitive (*ei*) transporters are resistant to NBMPR inhibition and retain almost full transport capability at NBMPR concentrations upto 1 μM (69, 70). These two transporter subtypes also differ in their sensitivities to the coronary vasodilators dipyridamole and dilazep (69). *Es* transporters from most species are potently inhibited by these drugs whereas *ei* transporters are considerably more resistant to these compounds. Rat *es* transporters are unique in that they are only poorly inhibited by dipyridamole (69).

Both equilibrative transporter subtypes show broad substrate specificities and transport purine and pyrimidine nucleosides with apparent affinities of 20-400 μM, although *ei* transporters in general have lower apparent affinities than *es* transporters for their substrates (69, 70).

Equilibrative nucleoside transporters from different species and even different cell types within species show considerable heterogeneity in terms of permeant affinities, turnover numbers and electrophoretic mobilities suggesting that different isoforms of this carrier might exist (69). The *es* type transporter from human erythrocytes has been extensively studied (69, 70, 73). This transporter has been

purified, reconstituted into phospholipid vesicles and polyclonal antibodies have been raised against it (74). The human erythrocyte *es* transporter is a glycoprotein with an apparent M_r of 55,000 that decreases to ~ 44,000 upon endoglycosidase F treatment (73). Biochemical studies mapped the site of N-linked glycosylation close to one end of the protein and photoaffinity labeling experiments with [^{3}H]NBMPR localized the site of NBMPR binding to within 16 kDa of this glycosylation site (73). Photoaffinity labeling studies with [^{3}H]NBMPR have also revealed that the differences in electrophoretic mobilities of *es* transporters from different species are largely attributable to differences in the oligosaccharide moieties of these proteins (73). Furthermore, the sites of [^{3}H] NBMPR photolabeling, carbohydrate attachment and trypsin cleavage are similar in *es* transporters from different species, suggesting that these permeases have common structural features (73). This conclusion is supported by the observation that polyclonal antibodies raised against the human erythrocyte *es* transporter cross react with transporters from other species including those from pig and rabbit erythrocytes and rat liver, in spite of differences in size and inhibitor sensitivities (74).

Es and *ei* transporters are often expressed in the same tissue. It is not clear why two transporter types with very similar transport characteristics should coexist, although there is some evidence that *ei* but not *es* transporters could mediate transport of the purine base hypoxanthine (69, 70).

7.1.2 Molecular characterization of Human Equilibrative nucleoside transporters

In the past few years the cDNAs for *es* and *ei* transporters from several different species and tissues have been cloned and characterized (75, 76, 77, 78, 79, 80, 81). The amino terminal sequence of the purified human erythrocyte nucleoside transporter was used to clone the cDNA for the human placental *es* type transporter, hENT1 (75). hENT1 is a 456 residue glycoprotein with 11 predicted transmembrane segments and three potential N-linked glycosylation sites (Figure 1-8) (75). Based on earlier observations that *es* transporters are glycosylated at one end of the protein, Asn 48 in the loop between TMs 1 and 2 was believed to be the only glycosylated site in hENT1 (73, 75). This identification was subsequently confirmed by the electrophoretic analysis of a mutant hENT1 protein in which Asn 48 was replaced with Gln (80). This N48Q mutant retained the capacity to transport thymidine, suggesting that glycosylation was not essential for the function of hENT1 (80). Interestingly, this mutant displayed a decreased affinity for NBMPR binding, suggesting that Asn 48 itself and/or the N-linked glycosylation was important for NBMPR binding (80).

The cDNA for hENT2, an *ei* type transporter, has also been cloned from human placenta and other tissues (76, 78). hENT2 is 46% identical to hENT1 in amino acid sequence and has a similar predicted topology with 11 transmembrane segments. hENT2 has two potential N-linked glycosylation sites in the loop connecting TMs 1 and 2 including one at Asn 48 (76, 78), and appears to be heterogeneously glycosylated at one or both of these sites (81).

The carboxy two-thirds of hENT2 is nearly identical to 36 kDa mouse and human HNP36 proteins, which are delayed–early proliferative response gene products induced by serum (76, 78, 82). These HNP36 proteins appear to be amino-truncated versions of the hENT2 protein. Mouse HNP36 has been localized to the nucleolus where its concentration increases after stimulation with mitogen, although its function is not known (82). The functional relationship between hENT2 and HNP36 is unclear.

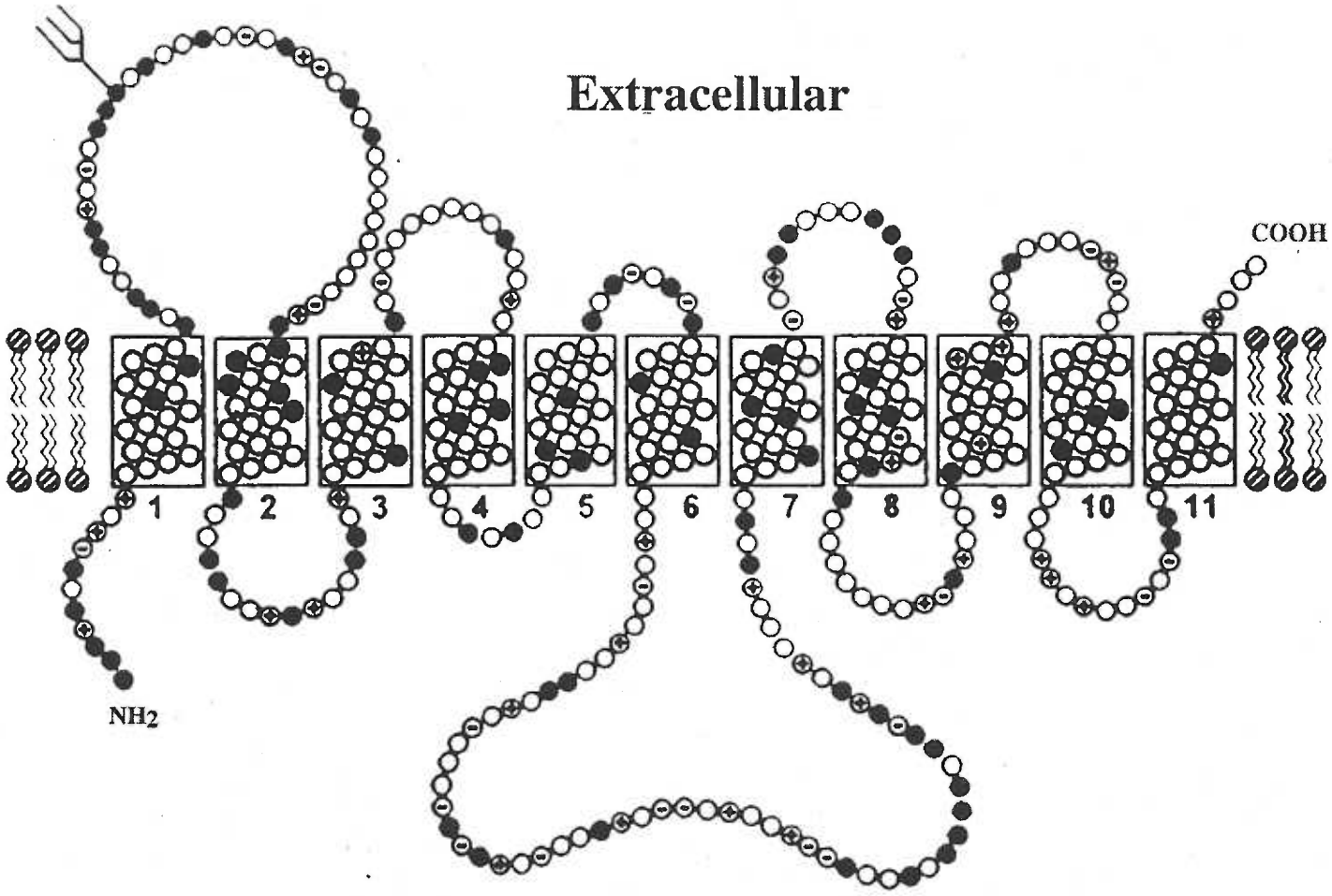
hENT1 and hENT2 display absolutely no sequence homology with the concentrative sodium-dependent nucleoside transporters (discussed below) and belong to a new transporter family, designated the equilibrative nucleoside transporter (ENT) family (72).

7.1.3 Functional characterization of hENT1 and hENT2

hENT1 and hENT2 have been expressed in *Xenopus laevis* oocytes (75, 76) as well as in other transport-deficient cell lines (78, 81). Both transporters have broad permeant specificities and mediate the sodium-independent uptake of purine and pyrimidine nucleosides. Although hENT1 and hENT2 have very similar apparent affinities for uridine, hENT2 generally has a lower apparent affinity for most other nucleosides compared with hENT1, especially cytidine and guanosine (78, 81). However, hENT2 was found to have a higher apparent affinity for inosine than hENT1. This observation is interesting in light of the fact that uridine transport by hENT2 but not hENT1 is inhibited by the purine nucleobase hypoxanthine, suggesting that hENT2 might play a role in hypoxanthine transport (78, 81). Like hENT1, hENT2 has a broad tissue distribution, but is most highly expressed in skeletal muscle (75, 78).

Figure 1-8. Topographical model of hENT1.

The potential membrane-spanning helices are numbered and the N-glycosylation site at Asn 48 is indicated. Figure from Griffiths, M. *et al.* 1997. *Nature Medicine* 3, 89-93.


Extracellular
COOH
1
2
3
4
5
6
7
8
9
10
11
NH2

It has been suggested that hENT2 might play a role in the efflux of inosine and hypoxanthine from skeletal muscle cells during periods of strenuous exercise when there is a net degradation of purine nucleotides (78). Functional hENT1 and hENT2 transporters have been identified in the nuclear envelopes of cultured human choriocarcinoma cells (83). These intracellular transporters could be involved in the transport of nucleosides from the cytosol into the lumen of the endoplasmic reticulum, where several enzymes of nucleotide biosynthesis are located.

7.1.4 Pharmacology

NBMPR potently inhibits hENT1-mediated transport with an apparent K_i value of 2 nM (75). In contrast, concentrations of NBMPR less than 1 μM have very little effect on hENT2 mediated transport (76, 78). Even at concentrations as high as 10 μM, NBMPR inhibited hENT2 mediated transport by only 70% (76). Previous studies have shown that *es* transporters are very sensitive to inhibition by the coronary vasodilators dipyridamole and dilazep, whereas *ei* transporters are relatively resistant (69). These pharmacological agents inhibit adenosine transport across the plasma membrane thus maintaining high extracellular concentrations of adenosine and potentiating its interaction with its receptors (70). Accordingly, hENT1 is almost completely inhibited by both compounds at concentrations of 1 μM (75). hENT2 mediated nucleoside fluxes are only poorly inhibited by low concentrations of dipyridamole and dilazep, but are susceptible to inhibition at concentrations greater than 10 μM (76, 78). The sensitivities of hENT1 and hENT2 to inhibition by NBMPR and dipyridamole have been extensively characterized in the transport deficient PK15NTD cell line stably

transfected with constructs encoding these transporters (81). These studies revealed an ~7000 fold difference in the sensitivities of hENT1 and hENT2 mediated uridine transport to NBMPR and an ~ 71 fold difference in their sensitivity to inhibition by dipyridamole (81). hENT1 mediated nucleoside influx is also inhibited by the anticancer nucleoside analogs cladribine, cytarabine, fludarabine and gemcitabine, suggesting that hENT1 may be involved in the uptake of these drugs (75). Both hENT1 and hENT2 are inhibited by the antiviral drug AZT and by 5-fluorodeoxyuridine (5-FdUrd), and hENT1 but not hENT2 is inhibited by AraC. Thus, these transporters likely play important roles in the cellular uptake of nucleoside analogs used in the treatment of a number of diseases.

7.1.5 Rat equilibrative nucleoside transporters

cDNAs encoding rat *es* (rENT1) and *ei* (rENT2) type nucleoside transporters have also been cloned from several tissues (77). rENT1 and rENT2 are 78% and 50% identical to hENT1 respectively and both have a predicted topology of 11 transmembrane segments (77). Both rat transporters have broad substrate specificities and transport purine and pyrimidine nucleosides (77). The cDNAs for *es* (mENT1) and *ei* (mENT2) nucleoside transporters have also been cloned from mouse tissues (84).

rENT1 differs most dramatically from hENT1 in its lack of sensitivity to inhibition by dipyridamole. hENT1 mediated uridine transport is potently inhibited by dipyridamole with an IC_{50} of 140 ± 2 nM (75, 77). In marked contrast, concentrations of dipyridamole up to 1 μM have no effect on rENT1 transport activity (77). Chimeras have been constructed between hENT1 and rENT1 in order to identify the

dipyridamole interacting site on the human transporter (85). These studies have revealed that the region including TMs 3-6 of hENT1 contribute the major site for dipyridamole binding (85). Since vasoactive drugs compete with NBMPR and nucleoside substrates for binding to the transporter, this region is likely to be localized within or close to the substrate binding site. Point mutations within this region should provide more detailed information about individual amino acids involved in interacting with both vasoactive drugs and with nucleoside substrates (85). Table 1-1 lists the properties of nucleoside transport systems from mammalian cells.

Table 1-1: Properties of mammalian nucleoside transporters

NT Process (acronym)	NT Process (Numerical)	Permeant Selectivity	NBMPR Sensitivity	NT Protein	Na^+:ligand Stoichiometry
Equilibrative Transporters					
Es		Purine & Pyrimidine Nucleosides	Sensitive	hENT1, rENT1, mENT1	
Ei		Purine & Pyrimidine Nucleosides	Insensitive	hENT2, rENT2, mENT2	
Concentrative Transporters					
cif	N1	Purine nucleosides, Uridine	Insensitive	hSPNT1/hCNT2 SPNT/rCNT2	1:1
cit	N2	Pyrimidine nucleosides, Adenosine	Insensitive	hCNT1, rCNT1	1:1
cit	N4	Pyrimidine nucleosides, Adenosine, Guanosine	Insensitive		1:1
cib	N3	Purine & Pyrimidine nucleosides	Insensitive	hCNT3, mCNT3	2:1
Cs	N5	Adenosine analogs	Sensitive		
Csg	N6	Guanosine	Sensitive		

7.1.6 The sodium dependent transporters: biochemical characterization

Sodium dependent transporters utilize the inward directed sodium gradient to actively transport substrates against their concentration gradients. Unlike equilibrative transporters, concentrative transporters are found only in specialized cell types such as intestinal, renal and choroid plexus epithelia, liver splenocytes and macrophages (70). There are several subtypes of concentrative nucleoside transporters that differ in their substrate specificities, tissue distributions and their sensitivities to inhibition by NBMPR (71). The *cit*, *cif* and *cib* transport processes are all resistant to inhibition by NBMPR (Table 1-1). The N1 or *cif* system has a preference for purine nucleosides but also transports uridine, while the N2 or *cit* system shows a preference for pyrimidine nucleosides but also accepts adenosine and adenosine analogs as substrates (69, 71). N1 and N2 constitute the two major sodium dependent nucleoside transport systems in mammalian cells. Human kidney brush border membranes have a single type of Na^+-dependent nucleoside transport system, N4, which has the properties of the pyrimidine selective N2 system, but transports guanosine in addition to adenosine (86). In contrast, rabbit renal epithelial cells possess N1 and N2 systems but lack the N4 transporter found in human kidney (86). The rabbit choroid plexus contains yet another type of Na^+-dependent nucleoside transport system (N3 or *cib*) that has a broad substrate specificity and transports both purine and pyrimidine nucleosides (87). Clearly there is considerable heterogeneity in the types and distribution of sodium-dependent transporters both between tissues and between different species. The transport mediated by the N1, N2, N3 and N4 systems is electrogenic with the net

translocation of a positive charge(s) during each transport cycle. However, there are differences in the Na^+/nucleoside coupling ratios. The N1, N2 and N4 systems display a Na^+/nucleoside stoichiometry of 1:1 (69, 86, 88,). The broad specificity N3 transporter from the rabbit choroid plexus is different in that it transports 2 Na^+ molecules for every nucleoside molecule (87).

In addition to the above, two less well characterized Na^+-dependent and NBMPR sensitive transport systems have been identified (71). One of these, *csg*, is a Na^+-dependent, NBMPR and dipyridamole sensitive, guanosine specific transporter, first characterized in acute promyelocytic leukemic cells (89). The second, *cs*, is believed to mediate the sodium dependent transport of certain adenosine analogs.

7.1.7 Molecular characterization of sodium-dependent nucleoside transporters.

Xenopus laevis oocytes lack endogenous nucleoside transport systems and have been extensively used to clone and functionally characterize nucleoside transporters from a variety of tissues and species. Oocytes microinjected with rat jejunal poly A^+ RNA expressed both *cif* and *cib* type transport activities (90). cDNAs for a number of *cit* and *cif* type transporters from several mammalian species and tissues have now been cloned. Functional expression screening of a rat jejunal cDNA library identified a nucleoside transporter cDNA encoding a *cit*-type transporter protein (rCNT1) with 648 amino acids and 13 predicted transmembrane domains (91, 92). rCNT1 showed about 27% identity with NUPC, a proton/pyrimidine nucleoside symporter from *E. coli* (91, 93). rCNT1 was expressed in the rat jejunum and kidney but not in other tissues,

consistent with previous observations of *cit* type transport only in kidney and intestinal epithelia (91).

Although rCNT1 transported pyrimidine nucleosides and adenosine, the transport of adenosine was much slower than that of uridine (94). Kinetic analyses revealed that while the apparent K_m for adenosine (27 ±7 μM) was very similar to that for uridine (37 ± μM), the adenosine V_{max} value was far lower than the uridine V_{max} (91, 94). Therefore, in spite of its relatively high apparent affinity, adenosine was a poor permeant of rCNT1. Experiments with oocytes preloaded with [^{3}H]uridine revealed that uridine efflux was greatly enhanced in the presence of extracellular uridine but greatly diminished by extracellular adenosine, suggesting that the rate of conversion of the rCNT1-adenosine complex from the outward facing conformation to the inward facing conformation was slower than that of the rCNT1-uridine complex (94). The sodium-permeant stoichiometry of rCNT1 was determined to be 1:1 for both adenosine and uridine (94).

rCNT1 mediated the uptake of AZT and ddC, drugs used in the treatment of AIDS, with K_m values of 0.49 mM and 0.51 mM respectively (91). Thus the CNT1 transporter could play a role in the intestinal absorption of these drugs at the concentrations at which they would be present during oral administration protocols (91). rCNT1 also mediated the uptake of the antiviral nucleosides zidovudine and zalcitabine and rCNT1 mediated thymidine transport was inhibited by the anticancer drugs cladribine and cytarabine, suggesting that these drugs might also be substrates for this transporter (71).

The cDNA for a human homolog of rCNT1, designated hCNT1 was cloned from the kidney (95). hCNT1 has 648 amino acid residues and is 83 % identical to rCNT1. The gene for hCNT1 is located on chromosome 15 (95). Like rCNT1, hCNT1 mediated the Na^+-dependent uptake of pyrimidine nucleosides, adenosine and the antiviral pyrimidine nucleoside drug AZT (95). hCNT1 was expressed in the human small intestine, kidney and liver (95).

The cDNA for a *cif* type transporter was first isolated from rat liver by expression cloning in *Xenopus oocytes* (96). SPNT is a 659 residue protein that is expressed in a variety of tissues including the spleen and liver, but absent from the kidney (96, 97). ^{3}[H]adenosine uptake mediated by SPNT was almost completely inhibited by the purine nucleosides inosine and guanosine and by uridine but only partially inhibited by thymidine and cytidine (96). Dipyridamole and azidothymidine had no effect on adenosine transport by SPNT (96). An almost identical transporter designated rCNT2 was cloned from rat jejunum (94). The predicted amino acid sequence of rCNT2 was identical to that of SPNT except for an alanine to glycine substitution at position 419 and an isoleucine to valine change at position 522 (94). Purine selective rCNT2 and pyrimidine selective rCNT1 are 64 % identical in amino acid sequence (91, 94, 96).

The human homolog of SPNT, hSPNT1, isolated from the kidney, is 81 % identical to its rat counterpart (97). Like SPNT, hSPNT mediated uptake of ^{3}[H]inosine was completely inhibited by adenosine, guanosine and uridine and only poorly by thymidine and cytidine (97). hSPNT1 had a much higher apparent affinity for inosine

(K_m = 4.5 ± 1.0 μM) than for uridine (K_m = 80 ± 10 μM), suggesting that transporters belonging to the *cif* family are primarily purine nucleoside transporters (97). The purine nucleoside transporter from human small intestine, hCNT2 is identical to hSPNT1 except for an arginine to serine change at position 75 in the amino acid sequence (98). Interestingly, while hSPNT1 is strongly expressed in the liver, no hCNT2 transcript has been detected in this tissue, suggesting that the genes for these transporters are regulated in a tissue specific manner (98). The gene for hCNT2 is also located on chromosome 15 (98).

Chimeras between rCNT1 and rCNT2 have helped identify domains involved in determining substrate specificity (99). When TMs 8-9 of rCNT1 were replaced with the corresponding TMs of rCNT2, rCNT1 was converted to a purine nucleoside specific transporter with substrate selectivity and inhibition profile similar to that of rCNT2 (99). Furthermore, when TM 8 alone of rCNT1 was replaced with that of rCNT2, rCNT1 lost its pyrimidine nucleoside selectivity and could now transport both purine and pyrimidine nucleosides. These results suggested that TMs 8-9 played an important role in substrate recognition, and that TM 8 was critical for maintaining the pyrimidine nucleoside specificity of rCNT1 (99). Subsequent studies revealed that serine 318 within TM 8 was essential in maintaining the pyrimidine nucleoside specificity of rCNT1 (100). When serine 318 was replaced with the corresponding residue of rCNT2 i.e. glycine, the mutant transporter transported both purine and pyrimidine nucleosides. However, this S318G mutant had a much lower apparent affinity for purine nucleosides and therefore kinetically preferred pyrimidine nucleosides as

substrates (100). Mutation of glutamine 319 to methionine (the corresponding residue in rCNT2) had no effect on the substrate specificity of rCNT1. Interestingly, the S318G/Q319M double mutant had a significantly higher apparent affinity for purine nucleosides than the S318G single mutant, and transported both purine and pyrimidine nucleosides without much bias (100). Since TM 8 is amphipathic, it could conceivably line the substrate permeation pathway, with its hydrophilic side facing the aqueous "channel". Serine 318, which is located in the center of the hydrophilic side, could act as a gating residue and sterically hinder the passage of purine nucleosides. Replacement of serine with the smaller glycine would increase the size of the channel and allow access to the bulkier purine nucleosides (100). This is however a hypothesis that needs experimental confirmation.

Similar chimeras have been constructed between the human transporters hCNT1 and hCNT2 with very similar results (101). The regions responsible for conferring substrate selectivity are largely located within the carboxy-terminal halves of the human transporters, with TMs 7-9 (TM 7 of the human transporter corresponds to TM 8 of the rat transporter) playing a major role (101). Replacement of serine 319 of hCNT1 with glycine (the corresponding residue in hCNT2) converted hCNT1 into a broad specificity transporter capable of accepting purine in addition to pyrimidine nucleosides (101). Interestingly, a broad specificity transporter cloned from the Pacific Hagfish (hfCNT) has a glycine in the corresponding position (101). The simultaneous replacement of residues S319, Q320, S353 and L354 of hCNT1 with the corresponding residues of hCNT2 converted hCNT1, a *cit* type transporter, into a *cif* type transporter

with a specificity for purine nucleosides (101). The cDNA for a broad specificity *cib* type transporter was recently cloned from both human (hCNT3) and mouse (mCNT3) tissues (102). When expressed in *Xenopus* oocytes, hCNT3 mediated the uptake of both purine and pyrimidine nucleosides as well as purine and pyrimidine anti-cancer and anti-viral drugs. The transport mediated by hCNT3 was electrogenic with a Na^+:uridine coupling ratio of 2:1. This stoichiometry is consistent with that previously obtained for the *cib* type transporter from the rabbit choroid plexus (87). As described above, the mutation of two adjacent pairs of residues in the TM 7-9 region of hCNT1 (Ser319/Gln320 and Ser353/Leu354) to the corresponding residues in hCNT2 (Gly313/Met314 and Thr347/Val348), converted hCNT1 from a pyrimidine nucleoside specific to a purine nucleoside specific transporter (101). Interestingly, the corresponding residues in hCNT3 are Gly340/Gln341 and Ser374/Val375. This broad specificity transporter therefore contains one member of each pair identical to the corresponding residue in hCNT1 and the other member identical to the residue in hCNT2 (102).

7.2 Nucleobase transport in mammals

Nucleobase transport across the plasma membrane is less well characterized than nucleoside transport is. However, two classes of nucleobase transporters have been identified biochemically, equilibrative and concentrative (69). Like nucleoside transporters, there are several distinct nucleobase transporters in different tissues that differ in their kinetic properties, substrate selectivities and in the case of concentrative transporters, the Na^+:ligand stoichiometries (69, 103). The human erythrocyte

transporter is the best characterized equilibrative nucleobase transporter. Although purine and pyrimidine bases are substrates for this transporter, purines have a higher apparent affinity than pyrimidines and cytosine is not transported at all (69). Nucleobase analogs such as 5-flurouracil, allopurinol and oxypurinol are substrates for the erythrocyte transporter as are the antiviral nucleoside analogs acyclovir and gancyclovir although the latter compounds are transported with much lower efficiencies than purine bases (69).

Concentrative nucleobase transport has been extensively characterized in pig LLC-PK$_1$ late proximal tubule cells (104), rabbit choroid plexus (103) and opposum kidney (OK) epithelial cells (105), in addition to various other tissues. Studies carried out on cultured LLC-PK$_1$ renal epithelial cells identified a Na^+/nucleobase transporter with a high apparent affinity for hypoxanthine (K_m = 1 μM) and a Na^+:hypoxanthine stoichiometry of 1:1 (69, 104). Selected purine and pyrimidine bases including uracil, 5-flurouracil, thymine and guanine were identified as substrates for this transporter on account of their ability to inhibit hypoxanthine transport. Adenine and xanthine had no effect on hypoxanthine transport. Although the LLC-PK1 transporter did not recognize nucleosides, both dipyridamole and dilazep were relatively potent inhibitors of Na^+-dependent hypoxanthine uptake. Opossum kidney epithelial (OK) cells possess both Na^+-dependent and Na^+-independent nucleobase transport activities (105). Na^+-dependent hypoxanthine transport was potently inhibited by uracil, thymine and guanine and moderately by xanthine, whereas adenine had virtually no effect. Unlike the LLC-PK$_1$ transporter, this permease transported both xanthine and oxypurinol. The

stoichiometry of Na^+: hypoxanthine transport was determined to be 1:1 (105). Only hypoxanthine, adenine and guanine appear to be ligands for the Na^+-independent transporter in these cells (105). A Na^+-dependent nucleobase transporter has been identified in the rabbit choroid plexus that is distinct from the OK and LLC-PK_1 transporters in terms of substrate selectivity and kinetics. This transporter has a lower apparent affinity for hypoxanthine (K_m = 31.1 μM). Hypoxanthine transport was inhibited by a range of purine and pyrimidine bases including adenine, guanine, xanthine, cytosine, thymine and uracil suggesting that they may all be substrates for this transporter. Unlike the other Na^+-dependent nucleobase transporters characterized thus far, the Na^+-dependent nucleobase transporter from the choroid plexus exhibited a Na^+: hypoxanthine stoichiometry of 2:1 (103). The genes for all these nucleobase transporters have yet to be cloned.

7.3 Nucleoside transport in Trypanosomatids

Nucleoside and nucleobase transporters situated on the parasite plasma membrane supply the parasite with preformed host purines and thereby perform the first step in the salvage process. As these transporters transport certain pyrazolopyrimidine analogs of purine substrates that are selectively toxic to the parasite (e.g. allopurinol riboside), they are also pharmacologically important (66).

7.3.1 Biochemical characterization of nucleoside transport in *T. brucei*

Like *Leishmania*, *T. brucei*, the causative agent of African sleeping sickness, exhibits a digenetic life cycle, living alternately within the digestive tracts of tsetse flies and in the bloodstream of mammalian hosts. Bloodstream forms of *T. brucei* have two

distinct purine nucleoside transport systems, P1 and P2 (106). The P1 system transports adenosine, inosine and guanosine, whereas P2 mediates the uptake of adenosine and the nucleobase adenine. Both transport systems have apparent affinities for adenosine in the sub-micromolar range (K_ms of 0.15 μM and 0.59 μM for P1 and P2 respectively). Perhaps most strikingly, the P2 transporter mediates the uptake of melarsoprol and pentamidine, both frontline drugs used in the treatment of late and early stage African sleeping sickness respectively (106, 107). The finding that a mutant parasite cell line that lacks P2 activity is resistant to both melarsen and pentamidine underscores the importance of this transporter in the uptake of these drugs. The main binding motif recognized by the P2 transporter is $H_2N\text{-}C(R_1)=N\text{-}R_2$, a motif also present within the melamine moiety of melarsoprol and in pentamidine (108, 109). It was demonstrated recently that the P2 motif can be used to target toxic polyamine analogs for interaction with the P2 transporter. All of these compounds inhibited adenosine uptake by the P2 transporter and some exhibited weak trypanocidal activity *in vitro* (110).

Unlike bloodstream trypanosomes, procyclic or insect stage parasites appear to possess only a single P1 type purine nucleoside transporter, capable of transporting adenosine, inosine and guanosine (111). As transport of nucleosides is coupled to the proton motive force and accompanied by an influx of protons into the cell, this transporter appears to be an active proton symporter.

The related trypanosome *T. cruzi* also has two nucleoside transport systems, one for the uptake of purine nucleosides adenosine, inosine and guanosine and the

other for pyrimidine nucleosides (112). Dipyridamole potently inhibits pyrimidine nucleoside transport in this parasite.

In addition to nucleoside transporters, several nucleobase transporters have been biochemically characterized in both procyclic and bloodstream forms of *T. brucei*. Procyclic trypanosomes possess a high affinity purine base transporter, H1 that also transports allopurinol and oxypurinol (113). This transporter also appears to be an electrogenic proton symporter that mediates the co-transport of protons with the purine base. A highly selective uracil transporter, U1, has been characterized in procyclic parasites, that mediates the proton dependent uptake of uracil (114). Of chemotherapeutic interest is the observation that the anti-neoplastic agent 5-fluorouracil is a potent inhibitor of U1 activity, suggesting that it may be a substrate for this transporter. Two nucleobase transport systems H2 and H3 have been characterized in *T. brucei* bloodstream forms (115). H2 has a broad specificity for purine and pyrimidine bases and also transports guanosine. H3 is specific for purine bases.

7.3.2 Nucleoside transport in *Crithidia.*

Crithidia luciliae is a non-pathogenic trypanosomatid parasite of the blowfly *Phaenicia sericata*. These parasites possess two distinct nucleoside transporters, one for adenosine and its analogs and the other for guanosine and inosine (116). They also possess a nucleobase transporter for the uptake of hypoxanthine and adenine. The related parasite *C. fasciculata* also expresses two high affinity purine nucleoside transporters (117). CfNT1 mediates the uptake of adenosine and pyrimidine nucleosides and CfNT2 transports the purine nucleosides inosine and guanosine.

7.3.3 Biochemical characterization of nucleoside transport in *L. donovani*.

The analysis of nucleoside transport in *Leishmania* was aided considerably by the isolation of mutant clones that were resistant to growth inhibition by cytotoxic nucleoside analogs. The mutant TUBA5 and FBD5 cell lines were isolated by chemical mutagenesis with N-methyl-N-nitroso-N'-nitroguanidine, followed by selection in the toxic adenosine and inosine analogs tubercidin and formycin B respectively (118). Biochemical analyses of these mutant clones revealed that the tubercidin-resistant TUBA5 parasites had lost the ability to transport tubercidin, adenosine and pyrimidine nucleosides, but transported inosine and guanosine normally. In contrast the FBD5 parasites were unable to take up formycin B, inosine or guanosine but retained adenosine transport capability (118). These studies suggested that there were two distinct nucleoside transport systems in *L. donovani* promastigotes, one (LdNT1) for the uptake of adenosine and pyrimidine nucleosides and the second (LdNT2) for the transport of inosine and guanosine. The presence of two transport systems for nucleoside uptake was further confirmed by biochemical studies in wild type parasites. Thus, inosine transport in wild type parasites could be inhibited by guanosine and formycin B but not by adenosine or uridine. Conversely, adenosine and uridine could inhibit adenosine uptake whereas inosine and guanosine had virtually no effect. Moreover, uridine competetively inhibited adenosine transport suggesting that these substrates shared a common permeation pathway (119).

Both LdNT1 and LdNT2 transport systems displayed high apparent affinities for their substrates. Inosine and adenosine were each transported with apparent K_m values of ~ 0.7 μM (119). As these values are about two orders of magnitude lower than the K_m values for purine nucleoside transport by mammalian equilibrative nucleoside transporters, *Leishmania* are able to compete effectively for purines in the host environment. NBMPR and dipyridamole, both potent inhibitors of mammalian *es* transporters, had no effect on LdNT1 or LdNT2 mediated transport (119).

Studies by Ogbunude *et al.* have revealed that different strains of *L. donovani* promastigotes express essentially the same inosine/guanosine transporter but have different adenosine transport characteristics, suggesting that multiple adenosine transporters might exist (120).

L. donovani amastigotes also possess two distinct nucleoside transporters (121). One of these transporters, T1, has the same substrate specificity as LdNT1 and transports adenosine and pyrimidine nucleosides. The second transporter designated T2 is a novel amastigote-specific permease that transports only the purine nucleosides adenosine, guanosine and inosine.

7.3.4 Regulation of purine uptake in parasitic protozoa.

In 1985, Gottlieb first demonstrated that the activity of the *Crithidia fasciculata* 3'-nucleotidase was regulated by the availability of purines in the culture medium (53). There was an approximately 1000-fold increase in the activity of this enzyme when parasites were transferred from adenosine replete media to media containing growth-limiting concentrations of adenosine. The activities of other nucleotide hydrolyzing

enzymes such as 5′-nucleotidase and non-specific acid phosphatase also increased under these conditions but to lower degrees. The increase in 3′-nucleotidase activity was inhibited by cycloheximide suggesting that protein synthesis was essential for this phenomenon (53). Similar studies performed with *C. luciliae* revealed that the increase in 3′-nucleotidase (3′-NT/NU) activity was accompanied by an increase in the 3′-NT/NU mRNA levels (122). Cells starved for adenosine expressed ~ 100-fold higher levels of 3′-NT/NU transcripts than adenosine replete cells. This upregulation was most likely achieved post-transcriptionally as the rate of transcription of the 3′-NT/NU gene was similar under purine replete and starved conditions (122).

The rates of purine nucleobase and nucleoside uptake are also regulated by extracellular purine concentrations (116, 117, 123, 124). The rate of adenosine uptake was approximately 7-fold higher in purine starved *C. luciliae* parasites compared with cells grown in purine replete media (123). Furthermore, the rates of hypoxanthine and adenine uptake increased almost 100-fold under purine limiting conditions. Interestingly, the mechanism of hypoxanthine uptake changed from simple diffusion under purine rich conditions to carrier-mediated transport under conditions of purine starvation (123). Rates of adenosine and hypoxanthine uptake in oocytes injected with poly A^+ mRNA from purine replete and starved *C. luciliae* were similar (125). These results suggested that the enhanced purine transport observed in purine starved *Crithidia* was not due to an increase in the levels of mRNAs for these transporters.

T. brucei procyclics respond to purine starvation by increasing the rate of hypoxanthine uptake by ~ 4.5-fold and that of adenosine uptake by ~ 3.5-fold (117).

Kinetic studies revealed that the K_m values for adenosine uptake were similar under both conditions but the V_{max} value increased ~ 3-fold in purine starved parasites. *T. brucei* express a single nucleobase transporter (H1) during the procyclic stage of their life cycle but two distinct nucleobase transporters (H2 and H3) in the bloodstream stage (113, 115). Under purine limiting conditions there was no observable increase in the activity of the procyclic H1 transporter. Interestingly, the enhanced uptake of hypoxanthine by *T. brucei* procyclic forms under these conditions was due the expression of the high affinity H2 transporter that under normal conditions is expressed only in the bloodstream stage of the parasite's life cycle (117). As the H2 transporter has a higher apparent affinity for most purine bases, it is far more efficient in scavenging trace amounts of purines when their availability is limiting.

Depletion of adenosine resulted in a 10-fold increase in adenosine uptake and a 6-fold increase in uridine uptake by *L. donovani* promastigotes (124). There was no change in the rate of guanosine uptake under these conditions. Thus, extracellular adenosine concentrations modulate the activity of LdNT1, the adenosine/pyrimidine nucleoside transporter but not of the inosine/guanosine transporter LdNT2.

All parasitic protozoa depend on purine salvage for their survival. The ability to coordinately increase the activities of 3'-nucleotidase, the enzyme that generates nucleosides extracellularly and purine transporters that mediate their uptake, enables these parasites to adapt to conditions of limiting purine availability that are not optimal for growth.

7.3.5 Molecular genetics of parasite nucleoside transporters

The genes coding for the adenosine-pyrimidine nucleoside transporter (LdNT1) from *L. donovani* were the first nucleoside transporter genes to be cloned from any parasite. The cloning of these genes and the characterization of their protein products constitute a significant portion of the work done in this thesis and are described in the next chapter.

The isolation of the *LdNT1* genes led to the identification and cloning of a gene encoding a P1-type nucleoside transporter (*TbNT2*) from *T. brucei* (126). *TbNT2* is a member of a multigene family containing a cluster of 6 closely related genes (*TbNT2-TbNT7*), all of which encode purine nucleoside permeases (Marco Sanchez, unpublished). P1-type nucleoside transporters have been detected biochemically in both procyclic (insect stage) and bloodstream (mammalian stage) forms of *T. brucei* (106, 111). Although *TbNT2* is expressed only in bloodstream parasites, one or more of the other genes in the *TbNT2* cluster could be expressed in procyclic parasites (126).

The gene for a P2-type transporter from *T. brucei* (*TbAT1*) was cloned by functional expression in a mutant yeast line (ade2) defective in purine biogenesis (127). Since yeast cells do not take up exogenous adenosine, expression of *TbAT1* in the ade2 mutants permitted their growth in media containing adenosine as the sole purine source. In addition to adenosine and adenine, TbAT1 also mediated the uptake of the anti-trypanosomal drug melarsen oxide. Moreover, *TbAT1* cloned from a melarsen oxide-resistant clone of *T. brucei*, STIB 777R, encoded a transporter that differed from the wild-type transporter at 6 amino acid positions and failed to mediate adenosine

uptake when expressed in the ade2 yeast mutants (127). Significantly, two of these amino acid substitutions were identified in the TbAT1 transporter of a *T. brucei gambisience* isolate from a patient who was refractory to melarsoprol treatment, demonstrating that mutations within nucleoside transporters could contribute to drug resistance (127).

The gene coding for the LdNT2 inosine/guanosine transporter from *L. donovani* was cloned by virtue of its ability to restore the capability of the transport-deficient FBD5 cell line to take up inosine and guanosine (128). LdNT2 mediated the uptake of inosine and guanosine with high apparent affinities (K_ms for inosine and guanosine of 0.3 μM and 1.7 μM respectively), but failed to transport adenosine or 2-aminopurine riboside, suggesting that the oxygen on C-6 of the purine ring was important in ligand recognition (128).

Nucleoside transporter genes from two different strains of *Plasmodium falciparum*, the causative agent of malaria, have been cloned. *PfNT1*, cloned from the W2 strain encoded a permease with a broad specificity for both purine and pyrimidine nucleosides but not nucleobases (129). This transporter had a high apparent affinity for adenosine (K_m = 13.2 μM) and like the human *es* transporters was sensitive to inhibition by the vasodilator dipyridamole (129). PfNT1 also exhibited the novel ability to transport L- in addition to D-sterioisomers of purine and pyrimidine nucleosides (129). The transporter cloned from the 3D7 strain of *Plasmodium falciparum* designated PfENT1 differed considerably from PfNT1 in terms of substrate specificities and affinities (130). Like PfNT1, PfENT1 mediated the transport of purine and pyrimidine

nucleosides but with much lower apparent affinities (Km = 0.32 mM for adenosine). Unlike PfNT1, PfENT1 also transported several purine and pyrimidine bases including cytosine and was insensitive to dipyridamole (130). Moreover, PfENT1 mediated the uptake of the nucleoside analogs used in cancer chemotherapy including fludarabine, cladribine and gemcitabine as well as the anti-viral nucleoside analogues AZT, ddC and ddI (130). PfNT1 and PfENT1 are identical in amino acid sequence except for position 385, which contains a Phe in PfNT1 and a Leu in PfENT1 (129, 130). It is not clear whether this single amino acid change could produce such dramatic differences in the biochemical properties of the two transporters.

The gene encoding the *Toxoplasma gondii* adenosine transporter (TgAT) was isolated by first tagging the locus using insertional mutagenesis and then using probes derived from sequences flanking the transgene insertion to isolate wild-type *TgAT* genomic and cDNA clones (131). TgAT is purine-selective, mediating the uptake of several purine nucleosides and bases but not pyrimidines. Adenosine uptake by TgAT was strongly inhibited by dipyridamole. Table 1-2 lists the properties of the cloned parasitic nucleoside transporters.

Table 1-2: Properties of parasitic nucleoside transporters

Organism	NT Protein	Permeant Selectivity
T. brucei	TbNT2-TbNT7	Adenosine, Inosine, Guanosine
T. brucei	TbAT1	Adenosine, Adenine, Melarsen oxide
L. donovani	LdNT1.1, LdNT1.2	Adenosine & Pyrimidine nucleosides
L. donovani	LdNT2	Inosine, Guanosine
P. falciparum (W2)	PfNT1	Purine & Pyrimidine nucleosides
P. falciparum (3D7)	PfENT1	Purine & Pyrimidine nucleosides & nucleobases

Parasitic nucleoside transporters mediate the uptake of preformed purines from the host, the first step in the purine salvage pathway. In doing so, these transporters perform an essential nutritional function for the parasite. Moreover, these transporters also mediate the uptake of certain nucleoside analogs such as allopurinol riboside that are selectively toxic to the parasite. The study of these transporters is therefore both biologically and pharmacologically important. The biochemical characterization of nucleoside transport systems in intact parasites is often complicated by the presence of multiple transport systems with overlapping substrate specificities. Furthermore, in order to understand the molecular mechanisms of transport as well as structure/activity relationships, the genes for each of these transporters must be cloned and independently expressed. Chapter 2 describes the cloning of the genes for the *L. donovani* adenosine-pyrimidine nucleoside transporter by functional complementation of a transport-deficient and drug-resistant *Leishmania* cell line (TUBA5). These genes are the first for any parasitic nucleoside transporter to be cloned. The cloned *LdNT1* genes were expressed in both homologous and heterologous expression systems and the encoded permeases functionally characterized. The cloning of these genes allowed the identification of the mutations in the TUBA5 cell line that led to drug-resistance. The identification of these mutations and the first steps towards probing structure/function relationships in the *Leishmania* adenosine/pyrimidine nucleoside transporter are described in chapter 3 of this thesis.

Chapter 2:

Cloning of *Leishmania* Nucleoside Transporter Genes by rescue of a transport-deficient mutant

(transport/nucleosides/purine salvage/drug resistance/leishmaniasis)

Gayatri Vasudevan*, Nicola S. Carter†, Mark E. Drew*§, Stephen M. Beverley‡, Marco A. Sanchez*, Andreas Seyfang*, Buddy Ullman†, and Scott M. Landfear*¶

Departments of *Molecular Microbiology and Immunology and †Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, OR 97201 and ‡Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110

Corresponding Author:

Scott M. Landfear

Tel: 503-494-2426

Fax: 503-494-6862

E-mail: landfear@ohsu.edu

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Abbreviations: LdNT1, L. donovani nucleoside transporter 1; LdNT1, the gene encoding LdNT1; ORF, open reading frame; UTR, untranslated region; S.D., standard deviation; bp, base pairs.

§Current Address: Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205

¶To whom reprint requests should be addressed.

Abstract

All parasitic protozoa studied to date are incapable of purine biosynthesis and must therefore salvage purine nucleobases or nucleosides from their hosts. This salvage process is initiated by purine transporters on the parasite cell surface. We have used a mutant line (TUBA5) of *Leishmania donovani* that is deficient in adenosine/pyrimidine nucleoside transport activity to clone genes encoding these nucleoside transporters by functional rescue. Two such genes, *LdNT1.1* and *LdNT1.2*, have been sequenced and shown to encode deduced polypeptides with significant sequence identity to the human facilitative nucleoside transporter hENT1. Hydrophobicity analysis of the LdNT1.1 and LdNT1.2 proteins predicted 11 transmembrane domains. Transfection of the adenosine/pyrimidine nucleoside transport-deficient TUBA5 parasites with vectors containing the *LdNT1.1* and *LdNT1.2* genes confers sensitivity to the cytotoxic adenosine analog tubercidin and concurrently restores the ability of this mutant line to take up [3H]adenosine and [3H]uridine. Moreover, expression of the *LdNT1.2* open reading frame in *Xenopus* oocytes significantly increases their ability to take up [3H]adenosine, confirming that this single protein is sufficient to mediate nucleoside transport. These results establish genetically and biochemically that both *LdNT1* genes encode functional adenosine/pyrimidine nucleoside transporters.

Introduction

Parasitic protozoa of the genus *Leishmania* are the etiological agents of leishmaniasis, a disease that affects an estimated 12 million people worldwide (132) and ranges from the disfiguring cutaneous form to fatal visceral leishmaniasis (133). Since current empirically identified drugs suffer from many deficiencies, including toxicity and resistance, it is important to identify unique biochemical targets that could be exploited for rational development of improved therapies. Perhaps the most striking metabolic discrepancy between parasites and their hosts is the purine pathway. Whereas most mammalian cells synthesize purines *de novo*, all parasitic protozoa studied to date are unable to synthesize purines (38) and consequently must rely on purine acquisition from their hosts for survival and growth. The first step in this salvage pathway involves the transport of these substrates across the parasite plasma membrane. Moreover, these purine transporters initiate the uptake of certain pyrazolopyrimidine analogs of hypoxanthine and inosine that are toxic to both *Leishmania* and *Trypanosoma* (66). These pyrazolopyrimidines, such as allopurinol, allopurinol riboside, and formycin B, are subsequently metabolized to the nucleotide level by the parasite metabolic machinery and incorporated into RNA, metabolic transformations that do not occur in mammalian cells (66). Both the essential nutritional function of these transporters and their roles in mediating the toxicities of well characterized antiparasitic agents provide compelling rationale to study these membrane permeases at the molecular level.

Biochemical and genetic studies have established that *L. donovani* parasites express two distinct nucleoside transporters with non-overlapping substrate specificities (119). One transporter mediates the uptake of adenosine and pyrimidine nucleosides and also transports tubercidin, a cytotoxic analog of adenosine, while the other transporter allows membrane permeation of guanosine, inosine and formycin B (119). Parasites deficient in either or both transport activities have been isolated by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine followed by selection in tubercidin or formycin B (118). The availability of these null mutants provided a functional strategy for cloning genes encoding each of these nucleoside permeases.

In the present study, we have transfected the adenosine/pyrimidine nucleoside transport-deficient TUBA5 cell line with a cosmid library containing inserts of *L. donovani* genomic DNA (134) and screened individual transfectants for restoration of tubercidin sensitivity. Several tubercidin-sensitive transfectants were isolated and shown to contain distinct cosmids with overlapping inserts. Analysis of one of these cosmids has led to the identification of two open reading frames (ORFs) encoding 491 amino acids, designated *LdNT1.1* and *LdNT1.2* that mediate restoration of tubercidin sensitivity and [3H]adenosine and [3H]uridine transport capabilities to TUBA5 cells. Furthermore, expression of this *LdNT1.2* ORF in *Xenopus* oocytes stimulates the uptake of [3H]adenosine in this heterologous system. These results establish that the LdNT1.1 and LdNT1.2 proteins are functional nucleoside transporters.

Materials and Methods

Growth of Parasites and Nucleic Acid Preparation. The DI700 (135) and TUBA5 (119) strains of *L. donovani* were cultured at 26°C in DME-L medium (135) containing 10% fetal calf serum. Isolation of genomic DNA and RNA and preparation of Southern and Northern blots were performed as previously described (136).

Transfection and Screening for Tubercidin Sensitivity. To screen for cosmids containing the adenosine/pyrimidine nucleoside transporter genes, 30 separate transfections were performed as described (137) on ~4 x 107 TUBA5 promastigotes using 10 μg of DNA from a cosmid library of genomic DNA from the Ld4 strain of *L. donovani* in the shuttle vector cLHYG (134). One day after transfection, parasites were plated onto 1% agar plates containing DME-L medium plus 10% fetal calf serum and 50 μg/ml hygromycin. After ~2 weeks incubation at 26°C, colonies were picked and transferred into 96-well microtiter plates containing DME-L and 50 μg/ml hygromycin. Following several days of growth, an aliquot from each well was inoculated into two replica microtiter wells, one of which contained 10 μM tubercidin. After 10-14 days of growth, replica microtiter plates were examined for cells that grew in medium lacking tubercidin but not in medium containing tubercidin. Cultures which tested positive by this screen were expanded, and the cosmids were isolated from the parasites by alkaline lysis (138). About 2000 transfectants were screened yielding 5 positive clones, one of which contained the cosmid designated T1E1.

To localize the *LdNT1* genes within the T1E1 cosmid, restriction fragments were subcloned into the plasmid shuttle vector pSNAR (139). For characterization of the

LdNT1.1 gene, the 7.5 kb *Hind* III/*Xba* I fragment 1 (Fig. 2-1) was subcloned into pSNAR. For construction of the vector containing the *LdNT1.2* ORF, the 3.5 kb *Xba* I/*Eco*R I fragment 2 (Fig. 2-1) was digested with *Not* I, which has a site 29 bp upstream from the initiation codon, and with *Sph* I, which has a site 68 bp downstream from the termination codon. This *Not* I/*Sph* I fragment was blunted (140) with T4 DNA polymerase and subcloned into the blunted *Eco*R I site of pSNAR. Each recombinant plasmid was transfected into TUBA5 cells, and transfectants were selected in 100 μg/ml G418 and then tested for tubercidin sensitivity as described above.

DNA and Deduced Amino Acid Sequence Analysis. Manual sequencing of both strands of the *LdNT1.2* ORF in the *Xba* I/*Eco*R I fragment 2 (Fig. 2-1) was performed on single stranded DNA using the SequiTherm EXCEL DNA Sequencing Kit (EpiCenter Technologies) according to the manufacturer's instructions. The *LdNT1.1* gene in the *Hind* III/*Xba* I fragment 1 (Fig. 2-1) was sequenced in both directions by the Oregon Health Sciences University Microbiology Core Facility using a model 377 Applied Biosystems automated fluorescence sequencer (Perkin Elmer). Cycle sequencing was performed with AmpliTaq FS DNA polymerase using dichlororhodamine dye-labeled terminators (Perkin Elmer). The Gap program from the University of Wisconsin Computer Genetics Group (141) was used for pairwise alignments of LdNT1.1 and LdNT1.2 with related mammalian nucleoside transporter sequences. Transmembrane segments were predicted using the TMpred software (142). The *LdNT1.1* and *LdNT1.2* nucleotide sequences have been submitted to the

GenBankTM/EMBL Data Bank with accession numbers AF065311 and AF041473 respectively.

Uptake Assays. Uptake of [3H]adenosine and [3H]uridine was assayed by incubation of parasites with radiolabel followed by centrifugation through a cushion of dibutyl phthalate as described (143). Uptake was measured for a range of substrate concentrations over a time course ranging from 0 to 12 sec for cells expressing *LdNT1.1* and from 0 to 60 sec for cells expressing *LdNT1.2*. Initial uptake rates at each concentration were determined by linear regression analysis over the linear portion of the time course. These data were fitted to the Michaelis-Menten equation by least squares analysis using the KaleidaGraph program (Synergy Software). For expression of *LdNT1.2* in Xenopus oocytes, cRNA was transcribed with T7 RNA polymerase from a linearized pL2.5 *Xenopus* expression vector (144) containing the *LdNT1.2* ORF, injected into oocytes, expressed for 6 days at 15°C, and assayed for uptake of 50 μM [3H]adenosine as described (143).

Results

Cloning of the *LdNT1* adenosine/pyrimidine nucleoside transporter genes by rescue of the tubercidin sensitivity phenotype. Since the TUBA5 cell line is deficient in adenosine/pyrimidine nucleoside transport and is consequently resistant to micromolar levels of the adenosine analog tubercidin (119), the LdNT1 nucleoside transporter genes were cloned by transfecting TUBA5 cells with a cosmid genomic library from *L. donovani* (134) and screening transformants for restoration of tubercidin sensitivity. One of five independent cosmids that rescued the wild type tubercidin sensitivity phenotype with concomitant restoration of [3H]adenosine transport capability (not shown), T1E1, was chosen for detailed analysis. A restriction map of the T1E1 cosmid is shown in Fig. 2-1. To determine the location of the LdNT1 transporter genes within the ~40 kb cosmid insert, restriction fragments were subcloned into the pSNAR vector (139) and transfected into TUBA5 cells. The resulting transfectants were then tested for sensitivity to 10 μM tubercidin. Two adjacent restriction fragments, a 7.5 kb *Hin*d III/*Xba* I fragment (fragment 1 in Fig. 2-1) and a 3.5 kb *Xba* I/*Eco*R I fragment (fragment 2 in Fig. 2-1), conferred both tubercidin sensitivity (Fig. 2-1) and [3H]adenosine transport function (not shown) to transfected TUBA5 cells, suggesting that each restriction fragment contained at least one copy of *LdNT1*.

Deduced amino acid sequence of the LdNT1.1 and LdNT1.2 nucleoside transporters. Partial sequence of the 7.5 kb *Hin*d III/*Xba* I restriction fragment 1 (Fig. 2-1) revealed an ORF of 491 amino acids (Fig. 2-2) that contained 11 predicted

hydrophobic transmembrane domains (142) and exhibited 33% amino acid identity with the human equilibrative nucleoside transporter hENT1 (75). Furthermore, the predicted topologies of LdNT1.1 and hENT1 are similar, including the conservation of a large hydrophilic loop between predicted transmembrane segments 6 and 7. Partial sequencing of the 3.5 kb *Xba* I/*Eco*R I restriction fragment 2 (Fig. 2-1) revealed a single copy of a closely related ORF, *LdNT1.2*, that differed from the *LdNT1.1* ORF at six amino acid positions, including the last 3 amino acids in the sequence (Fig 2-2, asterisks and legend). Genomic Southern blots probed with the *LdNT1.2* ORF (Fig. 2-3A) revealed hybridizing fragments that are consistent with the map in Fig. 2-1. Thus the *Eco*R I genomic digest produced a single band of >15 kb, and the *Hin*d III digest generated a single band of ~13 kb, confirming that all the *LdNT1* genes were contained within the ~11 kb *Hin*d III/*Eco*R I fragment (fragments 1 and 2 together, Fig. 2-1). The *Xho* I digest produced three hybridizing bands, one of ~3 kb corresponding to the *Xho* I fragment marked A in Fig. 2-1, one of ~5 kb corresponding to the *Xho* I fragment marked B in Fig. 2-1, and one of ~1 kb corresponding to the *Xho* I fragment marked C in Fig. 2-1. Finally, the DNA sequences of the *LdNT1* ORFs generated from fragments 1 and 2 both contain an *Xho* I site followed by a *Bgl* II site 122 bp downstream. These two closely linked *Xho* I and *Bgl* II sites occur at only one position within both fragments 1 and 2, confirming the existence of only two tandemly linked *LdNT1* genes.

Recently, the genes for another putative human equilibrative nucleoside transporter hENT2 (76), a homologous protein from mouse HNP36 (82), and two equilibrative nucleoside transporters from rat, rENT1 and rENT2 (85), have been

cloned and sequenced. LdNT1.2 shows 28.9% identity to mHNP36, 29.3% identity to rENT1, and 30.9% identity to rENT2. Hence, the LdNT1.1 and LdNT1.2 transporters are members of an equilibrative nucleoside transporter family that spans the evolutionary range from primitive eukaryotes such as *Leishmania* to humans.

Functional expression of the *LdNT1* genes in *L. donovani* and in *Xenopus* oocytes. To confirm that the LdNT1 polypeptides are functional nucleoside transporters, the *LdNT1.2* ORF alone was subcloned into the pSNAR vector and transfected into TUBA5 cells. Uptake assays using [3H]adenosine and [3H]uridine (Fig. 2-4A,B solid symbols) confirmed that the *LdNT1.2* ORF confers nucleoside transport activity when transfected into the adenosine/pyrimidine nucleoside transport-deficient TUBA5 cells. In contrast, TUBA5 cells transfected with the pSNAR vector alone took up only residual amounts of each nucleoside (Fig. 2-4A,B open symbols). Similarly, expression of the *LdNT1.1* gene also conferred the capacity to transport adenosine and uridine upon TUBA5 cells (Fig. 2-5A,B).

To confirm that the LdNT1.2 polypeptide alone is a functional nucleoside transporter, *LdNT1.2* cRNA was expressed in the heterologous *Xenopus* oocyte system. Oocytes injected with *LdNT1.2* cRNA transported significantly more [3H]adenosine than control oocytes injected with water (Fig. 2-4C), establishing that LdNT1.2 functions by itself as a nucleoside transporter.

Kinetic characterization of LdNT1.1 and LdNT1.2. Substrate saturation curves for adenosine (Fig. 2-5A,C) and uridine (Fig. 2-5B,D) were determined for TUBA5 cells transfected with the *LdNT1.1* and *LdNT1.2* constructs. Least squares fits to the

Michaelis-Menten equation of the data from at least 3 independent experiments yielded apparent K_m values (mean ± S.D.) for LdNT1.1 of 0.17 ± 0.09 μM (n=4) adenosine and 5.6 ± 1.8 μM (n=3) uridine and apparent K_m values for LdNT1.2 of 0.66 ± 0.15 μM (n=3) adenosine and 40 ± 11 μM (n=4) uridine. These results reveal that the LdNT1.1 transporter has significantly lower K_m values for both adenosine and uridine, compared to the LdNT1.2 transporter. Hence the six amino acid differences between the two permeases are likely to confer distinct kinetic properties upon each transporter.

Analysis of the *LdNT1* locus and of *LdNT1* transcripts in the DI700 wild type and the TUBA5 mutant cell lines. To determine whether the TUBA5 cell line had undergone a deletion or significant rearrangement at the *LdNT1* locus that resulted in loss of adenosine/uridine transport activity, we probed Southern blots of restriction digested genomic DNA from wild type DI700 (Fig. 2-3A) and TUBA5 cells (Fig. 2-3B) with the *LdNT1.2* ORF. For all eight restriction enzymes examined, the hybridization patterns were identical for DI700 and TUBA5 cells, indicating the absence of large deletions or visible rearrangements at this locus. Similarly, Northern blots of polyadenylylated RNA from DI700 or TUBA5 promastigotes (Fig. 2-3C, lanes 1,2) probed with the *LdNT1.2* ORF revealed a single transcript of ~3.5 kb that is expressed to equivalent levels in both strains, demonstrating that the mutant phenotype of the TUBA5 cells is not due to the absence of *LdNT1* transcripts. Northern blots hybridized with probes for the 3'-UTRs of the *LdNT1.2* gene (Fig. 2-3C, lanes 3,4) or the *LdNT1.1* gene (Fig. 2-3C, lanes 6,7) revealed a ~3.5 kb *LdNT1.1* transcript in both wild type and TUBA5 cells but did not detect any transcript for the *LdNT1.2* gene in either cell line. A

positive control containing *LdNT1.2* cRNA (Fig. 2-3C, lane 5) demonstrated that the *LdNT1.2* 3'-UTR probe was able to detect homologous transcripts, confirming that the absence of a signal in lanes 3 and 4 was due to the absence of appreciable levels of *LdNT1.2* transcript in these parasites. The organisms used in this study were cultured promastigotes, similar to the life cycle stage that lives within the gut of the sandfly vector. It is possible that the *LdNT1.2* transcript is expressed exclusively in the amastigotes, the life cycle stage that resides inside the phagolysosomes of the vertebrate host macrophages. However, in the promastigote stage of the parasite life cycle, the *LdNT1.1* RNA is the major stable transcript from the *LdNT1* locus. Additional studies will be required to determine the relative levels of each mRNA in amastigotes.

Discussion

In the present study, we have cloned the genes for the LdNT1.1 and LdNT1.2 adenosine/pyrimidine nucleoside transporters of *L. donovani* by rescuing the mutant phenotype of the adenosine/pyrimidine transport-deficient TUBA5 cell line. This is a powerful technique that may be applied to the cloning of genes for other transporters and proteins for which a strong negative genetic selection is available. Using this approach, we have also cloned genes encoding the LdNT2 guanosine/inosine transporter using the guanosine/inosine transport-deficient FBD5 cell line (manuscript in preparation). In addition, a similar approach has been used to clone several genes involved in biosynthesis of a major surface glycoconjugate (145,146) and a gene required for biogenesis of the glycosome (147), an organelle involved in glycolysis and other metabolic interconversions in kinetoplastid protozoa.

The LdNT1.1 and LdNT1.2 transporters are members of a family of permeases currently represented by several mammalian equilibrative nucleoside transporters and possibly other proteins from *Caenorhabditis elegans* and yeast (75). Our results demonstrate that, similar to families for other classes of transporters (148), this family is represented across a large phylogenetic range from primitive eukaryotes like *Leishmania* to humans. One advantage of obtaining sequences from a diverse array of family members is the potential to identify a limited number of residues that are conserved over a large phylogenetic distance and that may represent functionally critical amino acids. It should be possible to test the potential roles of such highly conserved amino acids by site-directed mutagenesis.

One notable difference between LdNT1.1 and LdNT1.2 compared to the mammalian equilibrative nucleoside transporters is the limited substrate specificity of the parasite transporters. Whereas LdNT1.1 and LdNT1.2 transport adenosine and pyrimidine nucleosides (119), most mammalian equilibrative transporters have broad substrate specificities that accept all of the nucleosides (85). Furthermore, although the LdNT1 transporters are related to other equilibrative nucleoside transporters, it is not yet clear whether they are facilitative transporters or whether they are active transporters that might utilize the strong proton electrochemical gradient across the parasite plasma membrane (31, 149) to concentrate nucleosides inside these purine requiring organisms. It is notable that the MIT myo-inositol transporter from *L. donovani* is a proton symporter (150), even though it is a member of a superfamily containing the mammalian facilitative glucose transporters (151). In addition, previous experiments (119) have revealed partial inhibition of nucleoside transport in *L. donovani* by proton ionophores such as carbonylcyanide m-chlorophenylhydrazone (CCCP). Whether the LdNT1 transporters are proton symporters will be investigated in future studies.

The genomic arrangement of the *LdNT1* genes (Fig. 2-1) reveals the presence of two closely related tightly linked genes. Tandemly repeated genes containing from two to dozens of identical or closely related members are common among the kinetoplastid protozoa such as *Leishmania* (152). However, the presence of an *LdNT1.1* and an *LdNT1.2* gene on each of two homologous chromosomes of this diploid organism suggests that all four genes may have been inactivated in the

adenosine/pyrimidine nucleoside transport-deficient TUBA5 cell line. One theoretical possibility is that the original mutation resulted in a deletion or rearrangement of a region containing both the *LdNT1.1* and the *LdNT1.2* genes that led to either a loss of both ORFs or to unstable *LdNT1* RNAs. However, the absence of detectable deletions or rearrangements at this locus in TUBA5 cells (Fig. 2-3B) and the presence of stable *LdNT1.1* transcripts in both mutant and wild type lines (Fig. 2-3C) render either of these explanations unlikely. Furthermore, the presence of the *LdNT1.1* RNA in the TUBA5 cells rules out mutations affecting transcription, RNA processing, or RNA stability.

There are several alternative explanations for the null transport phenotype of the TUBA5 cells. 1) Both the *LdNT1.1* and *LdNT1.2* genes could have obtained different point mutations generating either stop codons or functionally compromising missense mutations. The wild type alleles on the homologous chromosome might then have been converted to the mutant genotype by 'loss of heterozygosity', a phenomenon that has been experimentally demonstrated in both *L. major* (153) and *L. donovani* (154). 2) A mutation may have been introduced into one of the *LdNT1* genes initially and then rapidly disseminated throughout other members of the family. Such 'gene correction' events have been documented in mammalian cells (155). 3) A missense or nonsense mutation in a single *LdNT1* gene could generate a dominant-negative phenotype if the LdNT1 permeases function as oligomers. 4) The mutation in the TUBA5 cells that inactivates LdNT1 transport activity may not be in the *LdNT1* genes themselves but in another gene that affects transport activity. In this case, expression of exogenous

copies of the *LdNT1* genes would rescue the transport-deficient phenotype by suppression rather than by complementation. Although distinguishing between these or other hypotheses is beyond the scope of the present work, it should be possible ultimately to elucidate the TUBA5 genotype by cloning and sequencing the *LdNT1.1* and *LdNT1.2* genes from the TUBA5 cell line. Furthermore, if debilitating missense mutations are found within the *LdNT1* ORFs of TUBA5 cells, these results would validate a forward genetic approach for identifying residues in the LdNT1 transporters that are critical for function. It should then be possible to isolate and characterize numerous independent adenosine/pyrimidine nucleoside transport-deficient cell lines containing different missense mutations within the *LdNT1* locus.

The functional rescue strategy employed here for restoration of tubercidin sensitivity/nucleoside transport activity and another rescue protocol previously accomplished to isolate genes required for assembly of the glycosome in *L. donovani* (147) both involved screening >1,000 transfectants for restoration of a wild type phenotype in a mutant background. In the present study, ~1250 cosmids would constitute one genome equivalent (145). The success of these two large scale screens underscores the utility of this genetic strategy and suggests that similar screens could be designed to identify genes involved in a variety of biological processes among the kinetoplastid protozoa.

Along with a gene for a related transporter from *Toxoplasma gondii* (156), *LdNT1.1* and *LdNT1.2* represent the first genes for nucleoside transporters to be cloned from any parasite. The central role of these permeases in the uptake and salvage of

purines by these purine requiring parasites, and their potential involvement in the development of drug resistance (106, 119), underscores the importance of studying these intriguing membrane proteins at the molecular level. The cloned *LdNT1.1* and *LdNT1.2* genes can now serve as cornerstones for investigating the structure, function and pharmacological importance of these transporters.

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FIG. 2-1. Restriction map of the T1E1 cosmid.

The map at the top includes the entire ~40 kb T1E1 insert, while the expanded map below contains only the ~15 kb *Eco*R I restriction fragment. Restriction fragments marked by arrows and designated 1, 2, A, B, and C are described in the text. The open boxes designated *NT1.1* and *NT1.2* indicate the two *LdNT1* ORFs. The direction of translation of these ORFs is from left to right. The symbol "+" indicates that the relevant restriction fragment conferred sensitivity to 10 μM tubercidin when subcloned into the pSNAR shuttle vector and transfected into TUBA5 cells. The symbol "-" indicates that this restriction fragment did not confer sensitivity to tubercidin when transfected into TUBA5 cells. Symbols for restriction sites are as follows: R, *Eco*R I; H, *Hin*d III, X, *Xba* I; N, *Not* I; B, *Bgl* II; Xh, *Xho* I. The *Hin*d III, *Xba* I, *Bgl* II, and *Xho* I sites were mapped only within restriction fragments 1 and 2.

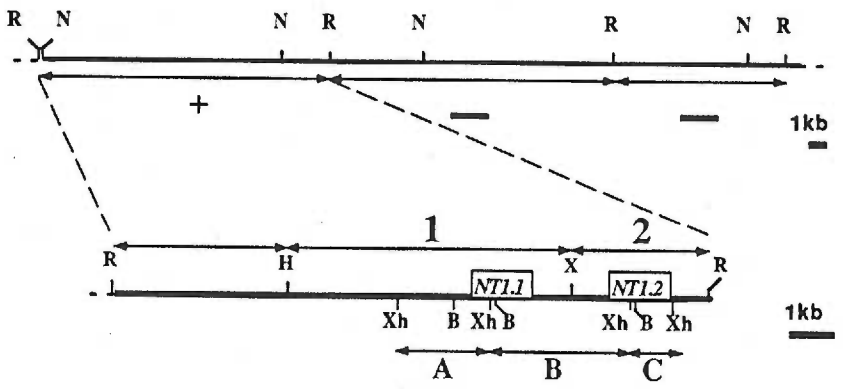

R N
N R N
R
N R
+
1kb
1
2
R
H
X
R
NT1.1
NT1.2
Xh B Xh B
Xh B Xh
1kb
A
B
C

FIG. 2-2. Alignment of LdNT1.1 with hENT1.

Deduced amino acid sequence of LdNT1.1 (top) compared to the human equilibrative nucleoside transporter (75) hENT1 (bottom) using the Pileup program (141) with a gap weight of 4 and a gap length weight of 3. Amino acids that are identical between the two sequences are shown in white over a black background. The black lines over the LdNT1 sequence and under the hENT1 sequence designate the predicted (142) transmembrane domains of each protein. The asterisks designate amino acids that are different in LdNT1.2. The amino acids P43, M107, T160, A489, T490, Y491 in LdNT1.1 are S43, I107, A160, E489, R490, H491 in LdNT1.2.

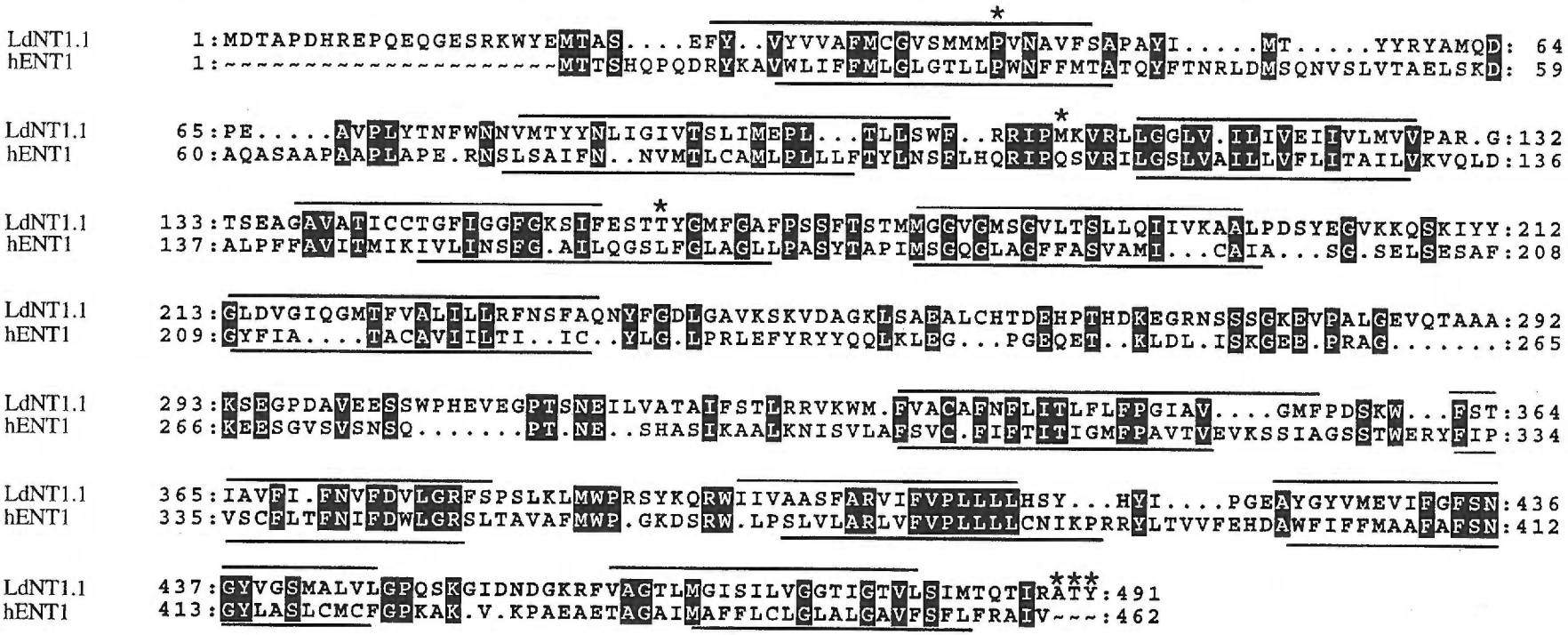
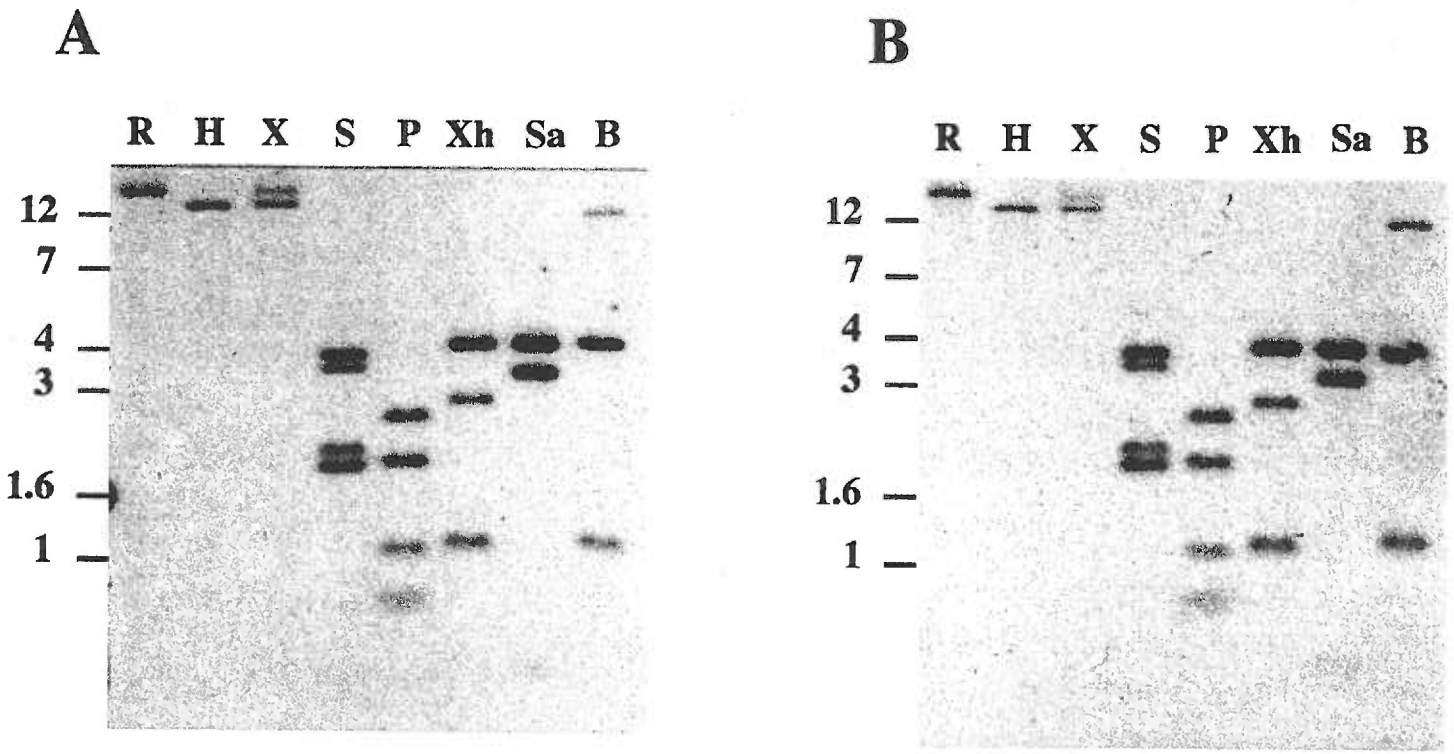

LdNT1.1 1:MDTAPDHREPQEQGESRKWYEMTAS....EFY..VYVVAFMCGVSMMMPVNAVFSAPAYI.....MT.....YYRYAMQD: 64
hENT1 1:~~~~~~~~~~~~~~~~~~~~~~MTTSHQPQDRYKAVWLIFFMLGLGTLLPWNFFMTATQYFTNRLDMSQNVSLVTAELSKD: 59
LdNT1.1 65:PE.....AVPLYTNFWNNVMTYYNLIGIVTSLIMEPL...TLLSWF..RRIPMKVRLLGGLV.ILIVEIIVLMVVPAR.G:132
hENT1 60:AQASAAPAAPLAPE.RNSLSAIFN..NVMTLCAMLPLLLFTYLNSFLHQRIPQSVRILGSLVAILLVFLITAILVKVQLD:136
LdNT1.1 133:TSEAGAVATICCTGFIGGFGKSIFESTTYGMFGAFPSSFTSTMMGGVGMSGVLTSLLQIIVKAALPDSYEGVKKQSKIYY:212
hENT1 137:ALPFFAVITMIKIVLINSFG.AILQGSLFGLAGLLPASYTAPIMSGQGLAGFFASVAMI...CAIA...SG.SELSESAF:208
LdNT1.1 213:GLDVGIQGMTFVALILLRFNSFAQNYFGDLGAVKSKVDAGKLSAEALCHTDEHPTHDKEGRNSSSGKEVPALGEVQTAAA:292
hENT1 209:GYFIA....TACAVIILTI..IC..YLG.LPRLEFYRYYQQLKLEG...PGEQET..KLDL.ISKGEE.PRAG.......:265
LdNT1.1 293:KSEGPDAVEESSWPHEVEGPTSNEILVATAIFSTLRRVKWM.FVACAFNFLITLFLFPGIAV....GMFPDSKW...FST:364
hENT1 266:KEESGVSVSNSQ.......PT.NE..SHASIKAALKNISVLAFSVC.FIFTITIGMFPAVTVEVKSSIAGSSTWERYFIP:334
LdNT1.1 365:IAVFI.FNVFDVLGRFSPSLKLMWPRSYKQRWIIVAASFARVIFVPLLLLHSY...HYI....PGEAYGYVMEVIFGFSN:436
hENT1 335:VSCFLTFNIFDWLGRSLTAVAFMWP.GKDSRW.LPSLVLARLVFVPLLLLCNIKPRRYLTVVFEHDAWFIFFMAAFAFSN:412
LdNT1.1 437:GYVGSMALVLGPQSKGIDNDGKRFVAGTLMGISILVGGTIGTVLSIMTQTIRATY:491
hENT1 413:GYLASLCMCFGPKAK.V.KPAEAETAGAIMAFFLCLGLALGAVFSFLFRAIV~~~:462

FIG. 2-3. Southern blots of genomic DNA and Northern blots of RNA from DI700 and TUBA5 cell lines.

Genomic DNA (5 μg) from the DI700 (A) and TUBA5 (B) lines was digested with the indicated restriction enzymes: R, *Eco*R I; H, *Hin*d III; X, *Xba* I; S, *Sac* I; P, *Pst* I; Xh, *Xho* I; Sa, *Sac* II; B, *Bgl* II. (C) Polyadenylylated RNA (4 μg) from the TUBA5 (lanes 1,3,6) and DI700 (lane 2,4,7) cell lines and 50 ng of *LdNT1.2* cRNA (lane 5) were separated on agarose-formaldehyde gels and transferred to a nylon membrane for hybridization. For each figure, the numbers at the left indicate the position of molecular mass markers with sizes given in kb pairs for (A) and (B) and kb for (C). Blots were probed with the *LdNT1.2* ORF (lanes 1,2), a 750 bp *Sph* I/*Xba* I fragment from the 3'-UTR of *LdNT1.2* (lanes 3,4,5), and a 1 kb *Nde* I/*Xba* I fragment from the 3'-UTR of *LdNT1.1* (lanes 6,7).


A
R H X S P Xh Sa B
12
7
4
3
1.6
1
B
R H X S P Xh Sa B
12
7
4
3
1.6
1

C

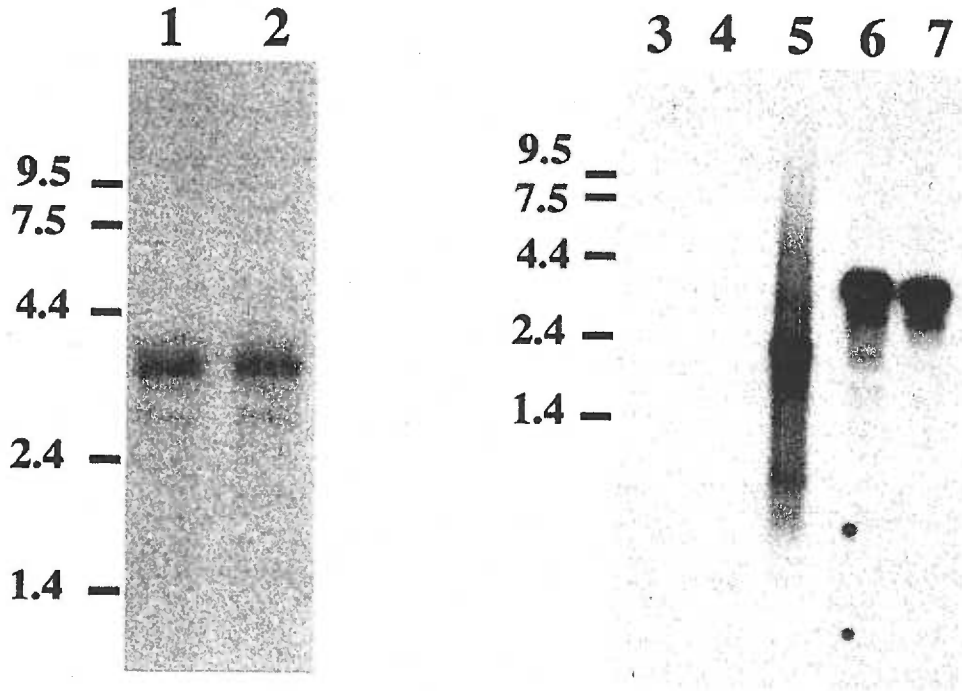
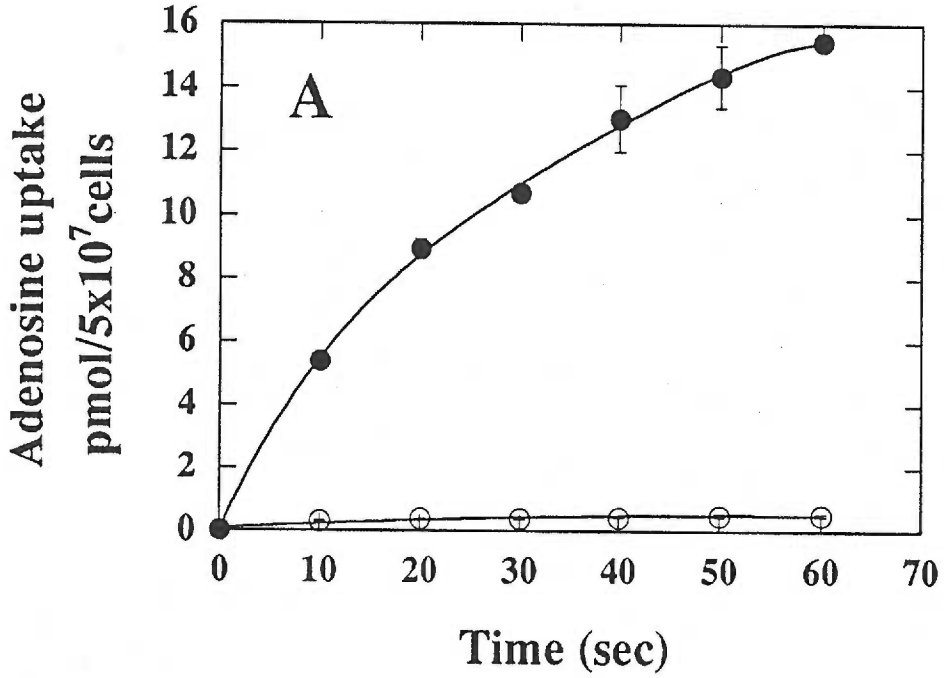
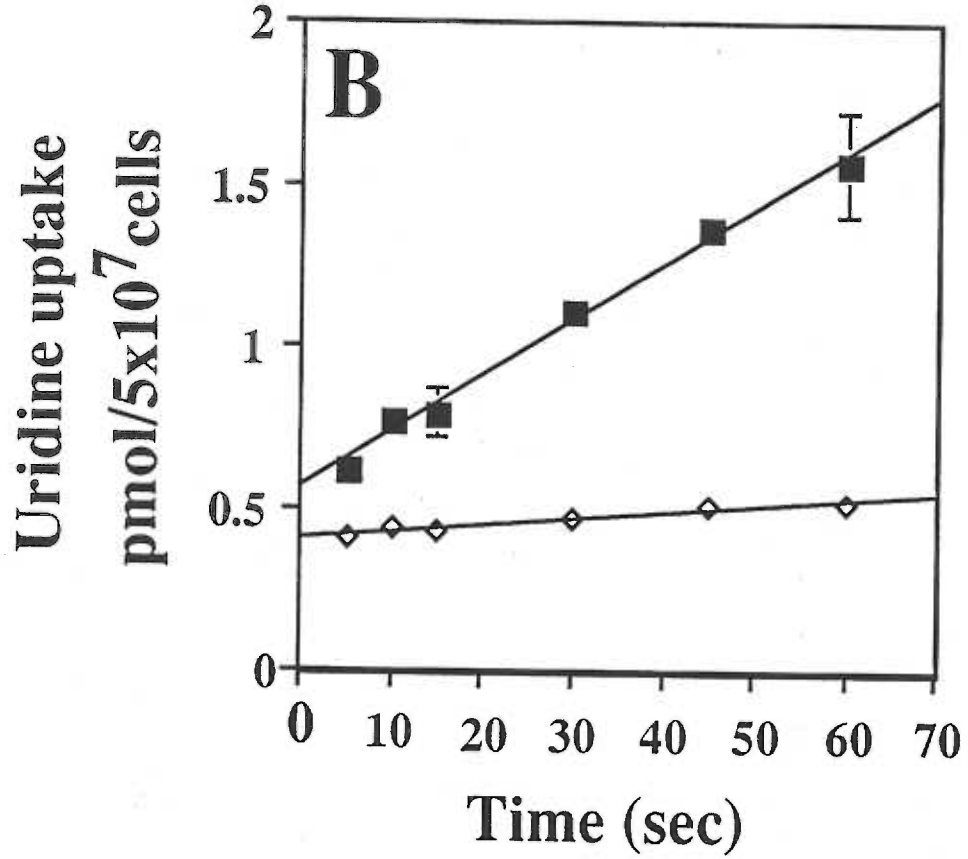


FIG. 2-4. Functional expression of LdNT1 genes in TUBA5 cells and in *Xenopus* oocytes.

TUBA5 cells transfected with the *LdNT1.2* ORF in the pSNAR vector (solid symbols) or with the pSNAR vector alone (open symbols) were assayed for uptake of 200 nM [3H]adenosine (A) or 1 μM [3H]uridine (B) (mean ± S.D., n = 2). For each time point, samples were assayed in duplicate, and the results were plotted as the mean and standard deviation (error bars). (C) *Xenopus* oocytes were microinjected with either water or ~25 ng of *LdNT1* cRNA, incubated at 15°C for 6 days, and assayed (143) for uptake of 50 μM [3H]adenosine over 1 h (mean ± S.D., n = 4).


A
Adenosine uptake
pmol/5x10^7cells
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
Time (sec)


B
Uridine uptake
pmol/5x10^7cells
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
Time (sec)

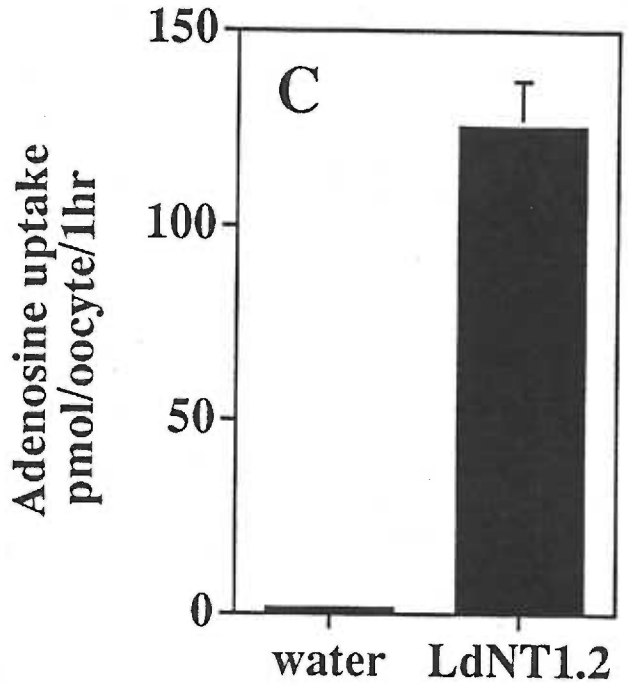
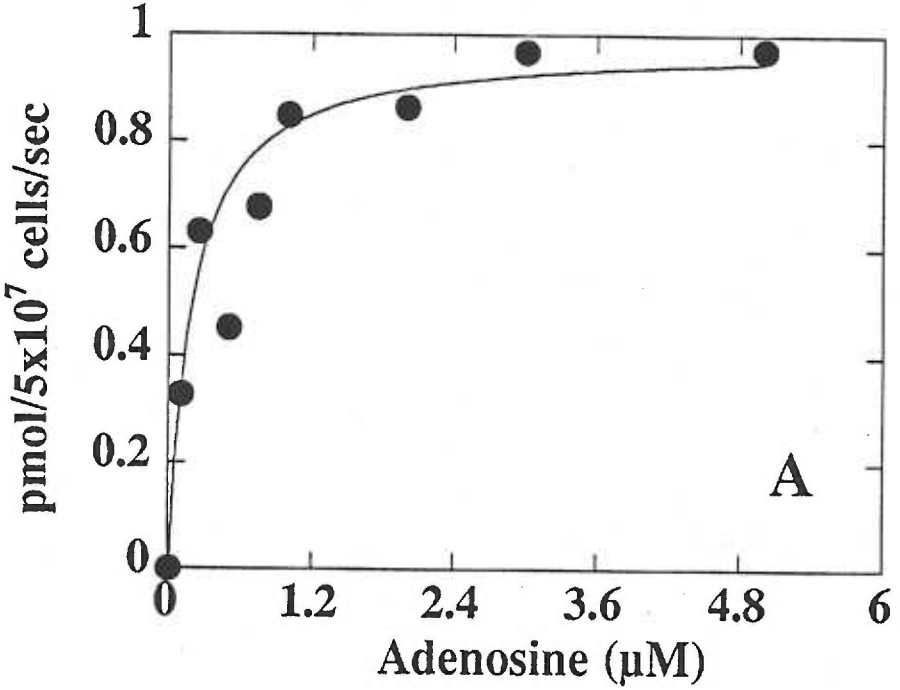
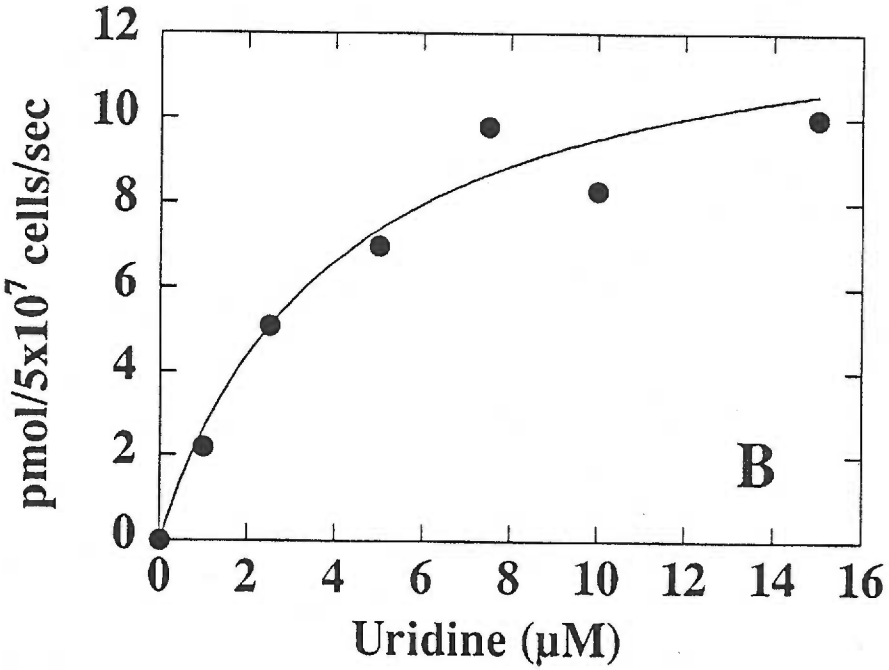
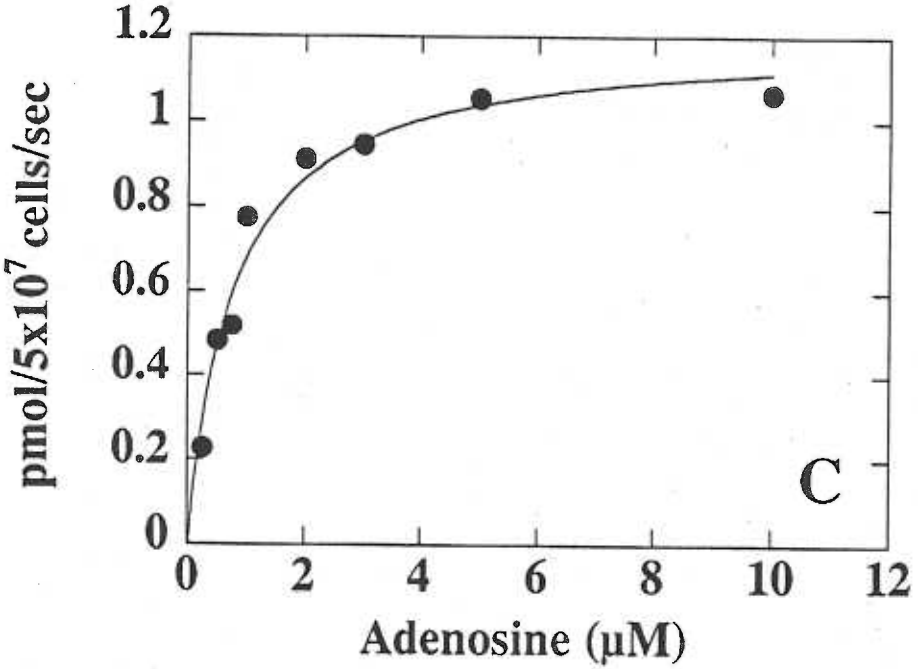

C
150
100
50
0
Adenosine uptake
pmol/oocyte/1hr
water
LdNT1.2

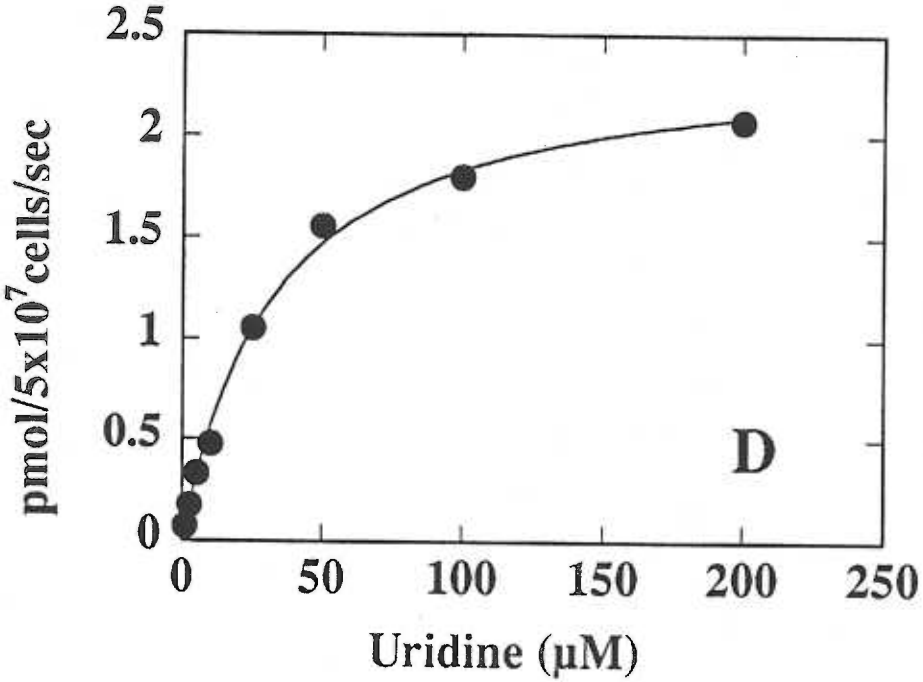
FIG. 2-5. Kinetic analysis of LdNT1.1 and LdNT1.2.

[3H]adenosine (A,C) and [3H]uridine (B,D) substrate saturation curves for TUBA5 cells transfected with constructs containg *LdNT1.1* (fragment 1 in Fig. 1) (A,B) and *LdNT1.2* (either fragment 2 in Fig, 1 or the LdNT1.2 ORF) (C,D) in the pSNAR vector.


pmol/5x10^7 cells/sec
1
0.8
0.6
0.4
0.2
0
0
1.2
2.4
3.6
4.8
6
Adenosine (μM)
A


12
10
8
6
4
2
0
pmol/5x10^7 cells/sec
0
2
4
6
8
10
12
14
16
Uridine (μM)
B


1.2
1
0.8
0.6
0.4
0.2
0
pmol/5x10^7 cells/sec
0
2
4
6
8
10
12
Adenosine (μM)
C


2.5
2
1.5
1
0.5
0
pmol/5x10^7cells/sec
0
50
100
150
200
250
Uridine (μM)
D

Chapter 3:

Point Mutations in a Nucleoside Transporter Gene from *Leishmania donovani* Confer Drug Resistance and Alter Substrate Selectivity

Gayatri Vasudevan*, Buddy Ullman† and Scott M. Landfear*‡

Departments of *Molecular Microbiology and Immunology and †Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, OR 97201

Corresponding Author:

Scott M. Landfear

Tel: 503-494-2426

Fax: 503-494-6862

E-mail: landfear@ohsu.edu

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Abbreviations: LdNT1.1, *L. donovani* nucleoside transporter 1.1; *LdNT1.1*, the gene encoding LdNT1.1; TM, transmembrane segment; ORF, open reading frame; GFP, green fluorescent protein; WT, wild type.

‡To whom reprint requests should be addressed.

Abstract

Leishmania parasites lack a purine biosynthetic pathway and depend on surface nucleoside and nucleobase transporters to provide them with host purines. *L. donovani* possess two closely related genes that encode high affinity adenosine-pyrimidine nucleoside transporters, LdNT1.1 and LdNT1.2, that transport the toxic adenosine analog tubercidin in addition to the natural substrates. In this study we have characterized a drug-resistant clonal mutant of *L. donovani* (TUBA5) that is deficient in LdNT1 transport and consequently resistant to tubercidin. In TUBA5 cells, the *LdNT1.2* genes had the same sequence as wild type cells. However, since *LdNT1.2* mRNA is not detectable in either wild type or TUBA5 promastigotes, LdNT1.2 does not contribute to nucleoside transport in this stage of the life cycle. In contrast, the TUBA5 cells were compound heterozygotes at the *LdNT1.1* locus containing two mutant alleles that encompassed distinct point mutations, each of which impaired transport function. One of the mutant *LdNT1.1* alleles encoded a G183D substitution in predicted TM 5 and the other allele contained a C337Y change in predicted TM 7. While G183D and C337Y mutants had only slightly elevated adenosine K_m values, the severe impairment in transport resulted from drastically (~20-fold) reduced V_{max} values. As these transporters were correctly targeted to the plasma membrane, the reduction in V_{max} apparently resulted from a defect in translocation. Strikingly, G183 was essential for pyrimidine nucleoside but not adenosine transport. A mutant transporter with a G183A substitution had an altered substrate specificity, exhibiting

robust adenosine transport but undetectable uridine uptake. These results suggest that TM 5 is likely to form part of the nucleoside translocation pathway in LdNT1.1

Introduction

Purine salvage pathways are critical to the survival of parasitic protozoa that lack the ability to synthesize the purine ring *de novo* (38). Parasite nucleoside transporters located on the plasma membrane perform the crucial function of transporting preformed purine nucleosides from the host into the parasite, the first step in the salvage process. These transporters are also relevant pharmacologically as they mediate the uptake of important anti–leishmanial agents such as allopurinol riboside that are analogs of the natural substrates (66).

Leishmania donovani promastigotes (the parasite form within the gut of the sandfly vector) possess two biochemically and genetically distinct nucleoside transport activities, one for the uptake of adenosine and pyrimidine nucleosides (LdNT1) (157) and the other for the transport of the purine nucleosides inosine and guanosine (LdNT2) (128). LdNT1 and LdNT2 also mediate the membrane permeation of the cytotoxic adenosine and inosine analogs, tubercidin and formycin B, respectively (118). A mutant *L. donovani* clone, TUBA5, that is deficient in LdNT1 transport activity has been isolated by mutagenesis of wild type parasites with N-methyl-N-nitroso-N'-nitroguanidine followed by selection in tubercidin (118). The TUBA5 cell line is incapable of transporting tubercidin, adenosine and pyrimidine nucleosides but transports inosine and guanosine normally (118).

The genes coding for the LdNT1 transporters were isolated by virtue of their ability to restore tubercidin sensitivity to the transport-deficient TUBA5 cell line (157). Two closely related and tandemly arranged genes, *LdNT1.1* and *LdNT1.2*, were

identified that encoded permeases with high apparent affinities for their substrate (157). While *LdNT1.1* was abundantly expressed in both wild type and TUBA5 promastigotes, there was no detectable *LdNT1.2* transcript in promastigotes of either cell type (157). Consequently, all of the nucleoside transport activity in promastigotes is contributed by the *LdNT1.1* genes. Since *L. donovani* is a diploid organism, both copies of *LdNT1.1* must either be inactive or encode non-functional proteins. Previously, it has been demonstrated that the TUBA5 line has not suffered any large deletion or rearrangement at the *NT1* locus that would disrupt the *LdNT1.1* and *LdNT1.2* ORFs (157). Furthermore, the presence of a full length *LdNT1.1* transcript in the TUBA5 promastigotes suggested that the mutations that lead to drug resistance did not interfere with either transcription, RNA processing or RNA stability (157).

The present study was undertaken in order to understand the genetic basis for the transport-deficient and drug-resistant phenotype of the TUBA5 cell line. While both *LdNT1.2* genes were wild type, single but distinct point mutations were identified within the ORFs of the two *LdNT1.1* genes that each produced functionally inactive transporters. One of these mutated *LdNT1.1* alleles encoded a transporter with a Gly-183→Asp (G183D) substitution in predicted transmembrane segment 5 (TM 5) and the other a transporter with a Cys-337→Tyr (C337Y) substitution in predicted TM 7. Both mutations dramatically lowered the V_{max} values of transport but had little effect on the apparent affinity of the transporter for adenosine. Interestingly, when Gly-183 was replaced by Ala the resulting permease retained the ability to transport adenosine but was severely impaired in its ability to transport the pyrimidine nucleoside uridine.

Thus, Gly-183 plays an important role in determining the substrate selectivity of LdNT1.1. These results suggest that a forward genetic approach can be a very powerful tool in identifying residues critical for permeation and for governing substrate specificity of nucleoside transporters.

Materials and Methods

Materials. [2,8,5-^{3}H]adenosine (54.4 Ci/mmol) and [5,6-^{3}H]uridine (39.5 Ci/mmol) was purchased from NEN™ Life Sciences Products Inc. Polyclonal anti-GFP antibodies were obtained from CLONTECH. All other chemicals and reagents were of the highest commercial quality available.

Parasite cell culture. The TUBA5 (118) strain of *L. donovani* was cultured at 26°C in DME-L (135) containing 10% fetal calf serum. TUBA5 transfectants were propagated in DME-L supplemented with either 100 μg/ml hygromycin B or G418. Genomic DNA was isolated from TUBA5 and DI700 strains as previously described (136).

Cloning and sequencing of *LdNT1.1* and *LdNT1.2* genes from TUBA5 parasites. Previous results (Gayatri Vasudevan, unpublished) indicated that both *LdNT1.1* and *LdNT1.2* genes were contained within a single ~17 kb *Hin*d III fragment. Therefore, a phage library of *Hin*d III-digested *L. donovan*i TUBA5 genomic DNA was constructed in the Lambda DASH II vector (Stratagene) according to protocols supplied by the manufacturer. The library was screened with the *LdNT1.1* ORF as probe, and positive clones were isolated using standard protocols (140). Phage DNA from two of these clones was isolated using the Phage Midiprep kit (Qiagen) and digested with *Eco*R I and *Xba* I to release a 7.5 kb fragment containing the *LdNT1.1* gene and a 3.5 kb fragment containing the *LdNT1.2* gene (157). Each of these fragments from both phage clones was subcloned into pBluescript for sequencing. *LdNT1.1* and *LdNT1.2* were then sequenced from both strands by the Oregon Health Sciences

University Microbiology Research Core Facility using a model 377 Applied Biosystems automated fluorescence sequencer (Perkin-Elmer).

Polymerase chain reaction (PCR) amplifications of the *LdNT1.1* and *LdNT1.2* ORFs from TUBA5 genomic DNA were performed using primers specific for each gene within the 5′ and 3 ′ untranslated regions and Pfu Turbo™ polymerase (Stratagene). All amplified fragments were subcloned using the Zero Blunt™ TOPO PCR Cloning kit (Invitrogen) and sequenced as described above.

Site-directed Mutagenesis and Construction of Plasmids. Mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) and wild type *LdNT1.1* subcloned in PCR^R-Blunt II-TOPO (Invitrogen) as template. Mutagenic primers were designed to incorporate both the desired mutation and a silent change to introduce a restriction site to facilitate screening. Mutagenized constructs were analyzed both by restriction digestion and sequencing. For expression in TUBA5 cells, wild type and mutated *LdNT1.1* genes were excised from PCR^R-Blunt II-TOPO with *Bam*H I and *Eco*R V and subcloned into the *Bgl* II/*Sma* I sites of the leishmanial expression vector pX63Hyg (158).

To generate N-terminal GFP fusions, ORFs of the wild type and mutated *LdNT1.1* genes were amplified by PCR using forward and reverse primers containing *Bam*H I restriction sites. Following restriction digestion, the ORFs were subcloned into the *Bam*H I site of the pXGGFP+2′ vector (159). All constructs were transfected into TUBA5 parasites as described (137).

Uptake Assays. Time courses of [^{3}H]adenosine and [^{3}H]uridine uptake were performed as reported (119). Briefly, parasites were incubated with radiolabeled substrate for various times and then centrifuged through a cushion of dibutylphthalate. For kinetic analysis, initial rates of uptake at each substrate concentration were determined by linear regression analysis over the linear portion of the time course. These data were fitted to the Michaelis-Menton equation by least-squares analysis using the Grafit program (Erithacus Software).

Preparation of Cell lysates and Immunoblots. Total cell lysates were prepared from TUBA5 parasites as described (160). Appropriate volumes of lysates containing comparable amounts of protein were mixed with equal volumes of Laemmli sample buffer (140), heated at 65° C for 5 min, separated on 8% SDS polyacrylamide gels and electroblotted onto nitrocellulose by standard methods (140). The blots were blocked with 5% non-fat dried milk in TBST (20mM Tris, pH 7.6, 150mM NaCl, 0.05% Tween 20) for 1 h and then incubated with the monoclonal anti-GFP antibody for 2 h. Blots were developed with a horseradish peroxidase-conjugated secondary goat anti-mouse (1:10,000) IgG and the chemiluminescence kit (Pierce) according to the manufacturer's instructions and exposed to XAR-5 film (Eastman Kodak Co.).

Fluorescence localization. For localization of the GFP tagged LdNT1.1 proteins, parasites were pelleted, washed in ice-cold phosphate buffered saline (PBS) and resuspended at a density of ~ 10^7 cells/ml. Cells were attached to poly-L-lysine coated coverslips and fixed with 100% methanol for 5 min. Coverslips were rinsed with PBS and mounted on slides in fluoromount-G (Fisher Scientific). Images were

acquired and deconvolved using the Deltavision Image Restoration System from Applied Precision.

Results

Cloning and Sequencing of *LdNT1* genes from TUBA5 parasites. In order to determine whether mutations within the *LdNT1* ORFs were responsible for the transport-deficient and tubercidin-resistant phenotype, *LdNT1.1* and *LdNT1.2* from the TUBA5 cell line were cloned and sequenced. The *LdNT1.1* ORF from one of two independently isolated genomic clones contained a single G→A transition at nucleotide position 548 and thus encoded a transporter with a G183D substitution in predicted TM 5 (Fig. 3-1). Interestingly the *LdNT1.1* ORF from the second clone contained a G→A transition at a different position within the ORF, nucleotide 1010, and encoded a transporter with a C337Y missense substitution in predicted TM 7 (Fig. 3-1). These results indicated that both *LdNT1.1* genes had acquired single but distinct point mutations resulting in transporters with non-conservative amino acid substitutions. In contrast, the *LdNT1.2* genes from both phage clones contained wild type sequences. In order to confirm these results, *LdNT1.1* and *LdNT1.2* were amplified from TUBA5 genomic DNA by PCR and sequenced. Four of the five independently amplified *LdNT1.1* genes encoded the C337Y mutation, while the fifth encoded the G183D substitution. All four independently amplified *LdNT1.2* genes had the wild type sequence.

Functional characterization of the G183D and C337Y transporters. To determine whether each of the G183D and C337Y mutations was sufficient to produce a nonfunctional transporter, each mutation was individually introduced into wild type *LdNT1.1* by site-directed mutagenesis and TUBA5 parasites transfected with these

mutagenized constructs were assayed for [^{3}H]adenosine uptake. While TUBA5 parasites expressing wild type LdNT1.1 exhibited robust uptake of radiolabeled adenosine, parasites expressing G183D and C337Y showed greatly reduced [^{3}H]adenosine transport capabilities (Fig. 3-2A & B). Moreover, whereas wild type LdNT1.1 restored the sensitivity of TUBA5 parasites to tubercidin, TUBA5 parasites overexpressing G183D and C337Y transporters continued to display the drug resistance phenotype (data not shown). These results confirmed that the G183D and C337Y mutations were responsible for both the transport-deficient and drug-resistant phenotype of the TUBA5 cells.

As both mutated transporters showed some residual transport activity when overexpressed in TUBA5 parasites, we were able to characterize them kinetically. G183D and C337Y mediated uptake of adenosine was saturable with apparent K_m values of 0.77 ± 0.13 μM (n = 2) and 0.9 ± 0.17 μM (n = 2) respectively. These values are somewhat higher than the K_m value of wild type LdNT1.1 (0.17 ± 0.09 μM) (157), but this modest increase in K_m cannot explain the transport-deficient phenotype. To determine whether the V_{max} values of the mutant transporters were reduced, GFP fusion proteins were constructed in which ORFs of wild type LdNT1.1, G183D and C337Y were fused to the carboxy terminus of GFP and expressed in the TUBA5 parasites. The results of substrate saturation experiments performed with the GFP fusion proteins are shown in Table 3-1. Consistent with earlier findings, GFP-G183D and GFP-C337Y displayed only slight increases in the apparent K_m values for adenosine compared with GFP-WT but significantly lower V_{max} values. Western blots

probed with a monoclonal anti-GFP antibody revealed that GFP-C337Y was expressed at slightly higher levels (1.5-fold) than GFP-WT, while GFP-G183D levels were about two-fold lower (data not shown). V_{max} values normalized to GFP fusion protein levels are shown in Table 3-1. Thus GFP-G183D and GFP-C337Y have ~18 and ~ 23 fold lower V_{max} values compared with GFP-WT respectively, confirming that the mutations primarily affect V_{max}.

Subcellular localization of GFP-WT, GFP-G183D and GFP-C337Y. The reduction in V_{max} values could reflect either the failure of G183D and C337Y to properly target to the cell surface or the innate impairment of transporter function. To determine whether the two mutants were correctly targeted to the cell surface, the subcellular locations of GFP-WT, GFP-G183D and GFP-C337Y were monitored using GFP fluorescence. As shown in Fig. 3-3A, GFP-WT was localized to the plasma membrane of the parasite cell body but excluded from the flagellum. A control TUBA5 cell line expressing GFP alone showed diffuse staining throughout the cytoplasm (data not shown). Like GFP-WT, both GFP-G183D and GFP-C337Y were targeted to the plasma membrane of the parasite (Fig. 3-3B & C). Furthermore, there was little or no staining of the endoplasmic reticulum and Golgi apparatus, indicating that these mutations did not cause substantial misfolding and retention within internal membranes. These results suggest that the G183D and C337Y mutations produced the TUBA5 phenotype by affecting some aspect of the transporter cycle itself.

Gly183 is a determinant of substrate selectivity. To analyze the function of G183 further, site-directed mutagenesis was used to replace this residue with Ala

(G183A) or Asn (G183N). TUBA5 parasites expressing G183A and G183N transporters were assayed for their ability to take up [^{3}H]adenosine. Fig. 3-2B shows that G183A transported adenosine robustly whereas the G183N mutant was severely impaired in transport. Thus, although G183 was not essential for adenosine transport, amino acids with bulky (G183N) and/or charged (G183D) side chains were not tolerated at this position. Similarly, a C337S mutant was capable of transporting adenosine, albeit at lower rates than WT, suggesting that C337 *per se* was not essential for function, whereas the C337F mutation completely abolished transport indicating that size restrictions operated at this amino acid position as well (data not shown).

The G183A mutant was also assayed for [^{3}H]uridine transport capability. Unexpectedly, G183A failed to transport [^{3}H]uridine significantly above background levels (Fig. 3-4A). To determine whether this inability to transport uridine stemmed from diminished uridine binding, the ability of unlabeled uridine at a 160-fold excess to inhibit [^{3}H]adenosine uptake mediated by wild type LdNT1.1 and G183A was investigated. As shown in Fig. 3-4B, [^{3}H]adenosine (0.25 μM) uptake by wild type LdNT1.1 was substantially reduced by unlabeled uridine (40μM). In marked contrast, unlabeled uridine at this concentration failed to significantly inhibit G183A mediated [^{3}H]adenosine uptake, confirming that the G183A mutation impaired the ability of the transporter to bind uridine. These results strongly indicated that G183 was essential for uridine but not adenosine transport.

Discussion

The transport-deficient and tubercidin-resistant TUBA5 cell line was isolated from wild type *L. donovani* by chemical mutagenesis followed by selection in drug (118). Biochemical characterization of this mutant cell line revealed normal levels and activities of intracellular purine salvage enzymes, suggesting that tubercidin resistance was due to impaired transport of the drug across the plasma membrane (118). This study has revealed that the molecular basis for this drug-resistant phenotype is point mutations within the two copies of the genes encoding the LdNT1.1 adenosine/pyrimidine nucleoside transporter that impair transport capability. Both G183D and C337Y permeases transported adenosine at very low levels when overexpressed in TUBA5 parasites. Kinetic analyses revealed that this impaired transport resulted from an ~20-fold decrease in V_{max} values for adenosine compared with wild type LdNT1.1. As both mutant transporters were correctly targeted to the plasma membrane, this reduction in V_{max} likely resulted from a defect in the translocation cycle itself. Not surprisingly, there were no mutations within the ORFs encoding the LdNT1.2 transporter. Since these genes are not expressed at detectable levels in wild type and TUBA5 promastigotes (157), there would be no strong selection pressure in the presence of tubercidin to acquire inactivating mutations.

To probe further the role of these residues in LdNT1.1 function, several C337 and G183 mutants were analyzed for transport proficiency. While neither residue *per se* was essential for adenosine transport, replacement of either with bulky residues (G183N and C337F) essentially abolished transport. These results also suggested that

the loss of function of G183D in the TUBA5 cell line was not solely due to the introduction of a negative charge into TM 5 but could be attributed at least partly to the bulkier Asp side chain. The replacement of Gly with Ala (G183A) was well tolerated in terms of adenosine transport capability. Although G183A mediated adenosine transport occurred at a lower rate than wild type, it was still significantly higher (~ 40-fold) than background rates (Fig. 3-2A, B). In marked contrast, G183A failed to transport uridine significantly over background (Fig.3-4A). Competition experiments revealed that a 160-fold excess of unlabeled uridine significantly inhibited wild type but not G183A mediated adenosine transport (Fig. 3-4B), suggesting that this mutant was impaired in its ability to bind uridine. Clearly, G183 is essential for pyrimidine nucleoside transport by LdNT1.1.

The absence of any structural information makes it is difficult to predict the mechanism by which G183 influences substrate selectivity. Glycine residues contribute extensively to helical packing interactions in membrane proteins (161, 162). Sequence motifs containing a pair of glycine residues located three residues apart (GXXXG) have been found to mediate high affinity associations, and these motifs occur frequently in transmembrane segments of membrane proteins (163, 164). Glycine residues in these motifs promote interhelical associations by providing a flat surface against which side chains of other residues can pack (164). Glycine also imparts flexibility to the polypeptide chain, a property that is often essential for function (163). For instance, conserved glycine residues in TM V of the *Escherichia coli* lactose permease are believed to contribute to the conformational flexibility of the substrate binding site (165).

The helical wheel diagram of the LdNT1.1 TM 5 shows that it is distinctly amphipathic, with G183 located approximately in the center of the hydrophilic face (Fig. 3-5). This location of G183 makes its involvement in interhelical packing interactions less likely. On the other hand, amphipathic helices often line substrate permeation pathways in transporters, with the hydrophilic residues forming specific interactions with substrates (100, 166, 167). Therefore, it is plausible that G183 lies within the substrate permeation pathway in LdNT1.1, particularly given its role in determining substrate selectivity. There are four glycine residues in TM 5 of LdNT1.1 (Fig. 3-1). It is possible that these residues provide conformational flexibility to TM 5 that is necessary for nucleoside binding and translocation or that G183 is part of a substrate binding pocket that cannot accommodate bulkier side chains. There are several examples of glycine residues contributing to a substrate or ligand binding pocket. Gly-121 in rhodopsin, for example, is part of the retinal binding pocket and is believed to form a cavity for the packing of the C9 methyl group of retinal (168, 169). Replacement of this residue with bulkier side chains increases steric interactions with the chromophore and alters the spectral properties of the mutants (168, 169). Similarly, three transmembrane glycine residues in the Ca^{2+}-ATPase appear to play important roles in Ca^{2+} binding and/or translocation (170). Alteration of C337 to a bulky tyrosine or phenylalanine virtually eliminates activity, whereas smaller side chains can be accommodated without eliminating transport. The fact that C337 is located in a hydrophilic patch of a largely hydrophobic helix (Fig. 3-5) suggests that this amino acid might also line the permeation pathway. Future studies using chemical

modification approaches such as the 'substituted cysteine accessibility method' (166) should permit an evaluation of this potential explanation for these two loss of function mutations.

Multiple sequence alignments indicate that only sixteen amino acid residues are conserved among all human and parasitic nucleoside transporters (171). These residues are likely to be important for transporter structure and/or function and are the obvious candidates for analysis by reverse genetic strategies. However, G183 and C337 are not among these highly conserved residues. That a forward genetic strategy led to the identification of these amino acids as important in LdNT1.1-mediated nucleoside permeation underscores the usefulness of this approach in structure/function analysis. Such forward genetic approaches offer a powerful tool to identify residues that are critical for substrate selectivity and/or permeation but that are not conserved among all the members of a transporter family. The isolation of multiple transport-deficient mutants with distinct mutations within the nucleoside transporter genes should allow a detailed analysis of structure/function relationships in these parasite transporters.

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FIG. 3-1. Location of missense mutations in the TUBA5 LdNT1.1 transporters. The positions of the G183D and C337Y mutations in the LdNT1.1 transporters encoded by the two mutant alleles of the TUBA5 cell line are indicated. The lines below the sequences indicate the positions of the predicted transmembrane segments (157).

LdNT1.1 (MUTANT ALLELE 1)

D

169 SSFTSTM**MGGVGMSGVLTSLLQIIVKAA**LPDSYE

TM 5

LdNT1.1 (MUTANT ALLELE 2)

Y

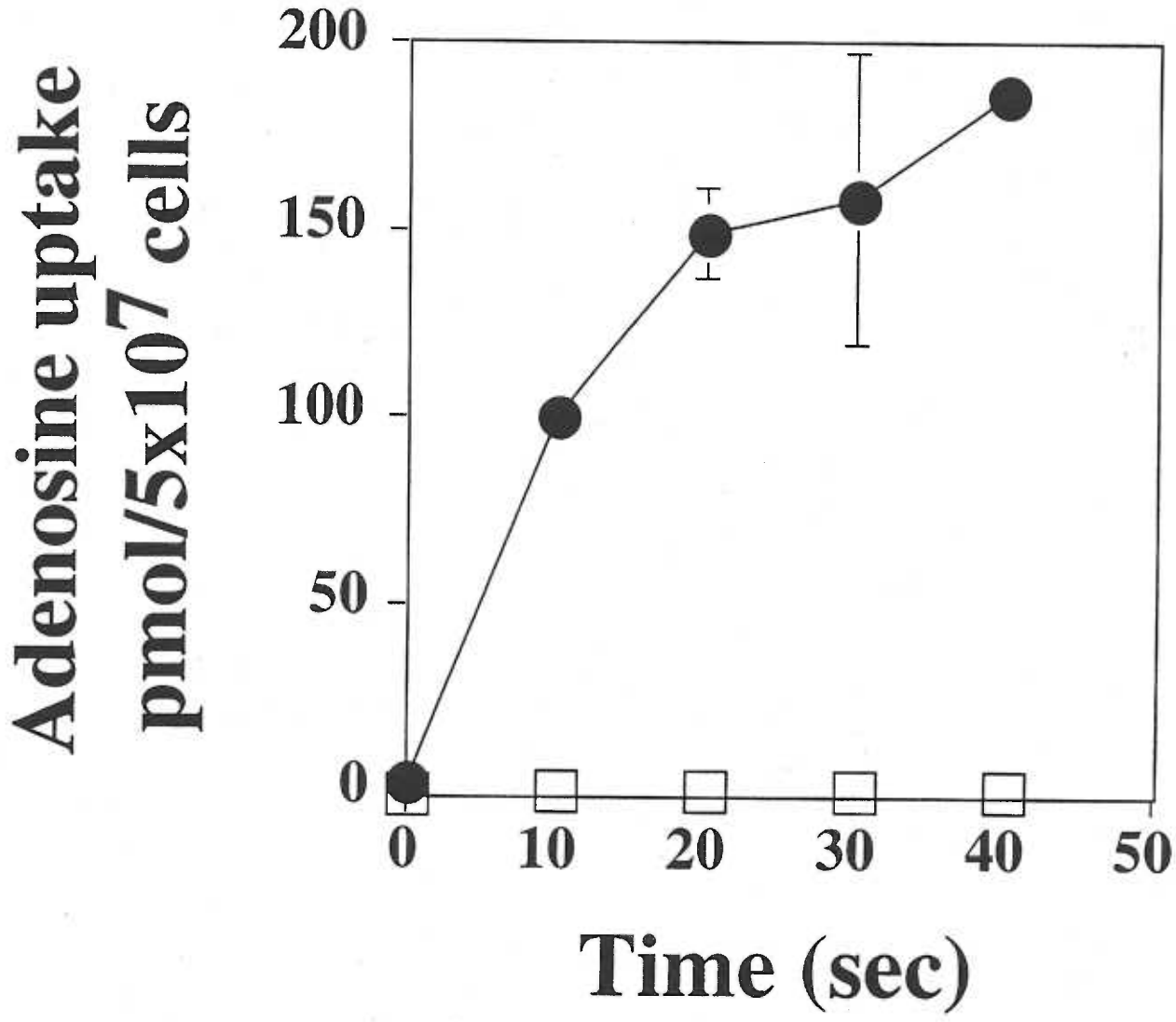
328 RRVKVM**FVACAFNFLITLFLFPGIAVGM**FPDSKWF

TM 7

FIG. 3-2. Functional characterization of G183 and C337 mutants.

TUBA5 parasites expressing (A) WT LdNT1.1 (●) or vector pX63Hyg (□) and (B) G183A (▲), G183N (☆), G183D (◆), C337Y (O) or pX63Hyg (□) were tested for uptake of 1 μM [^{3}H]adenosine. Results are expressed as mean ± S.D. (n = 2).

A


Adenosine uptake
pmol/5x10^7 cells
200
150
100
50
0
0
10
20
30
40
50
Time (sec)

B

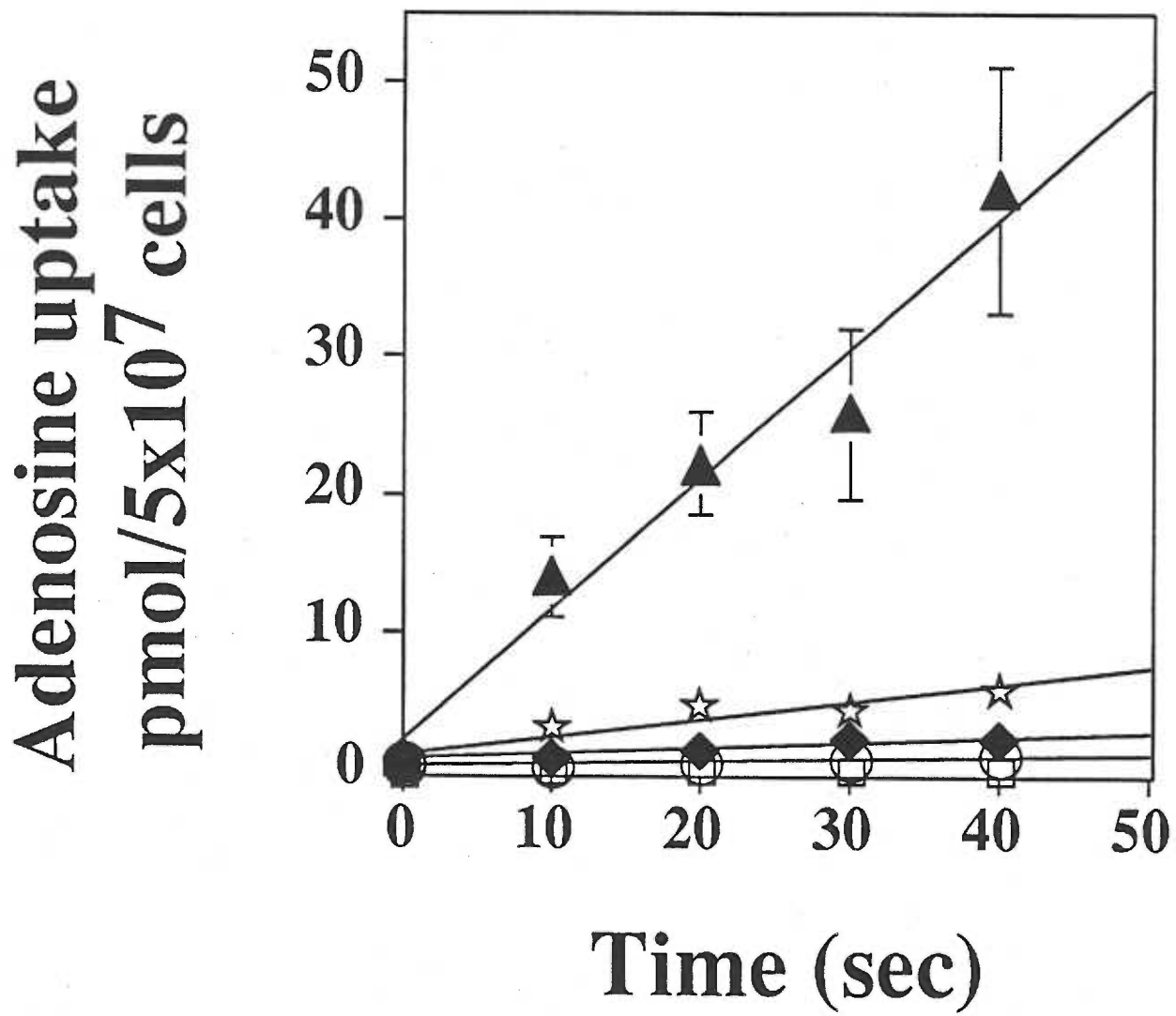

Adenosine uptake
pmol/5x10^7 cells
50
40
30
20
10
0
0
10
20
30
40
50
Time (sec)

FIG. 3-3. Subcellular localization of GFP-WT, GFP-G183D and GFP-C337Y.

Deconvolved fluorescence images of TUBA5 parasites overexpressing (A) GFP-WT, (B) GFP-G183D and (C) GFP-C337Y.

A
WT
LM

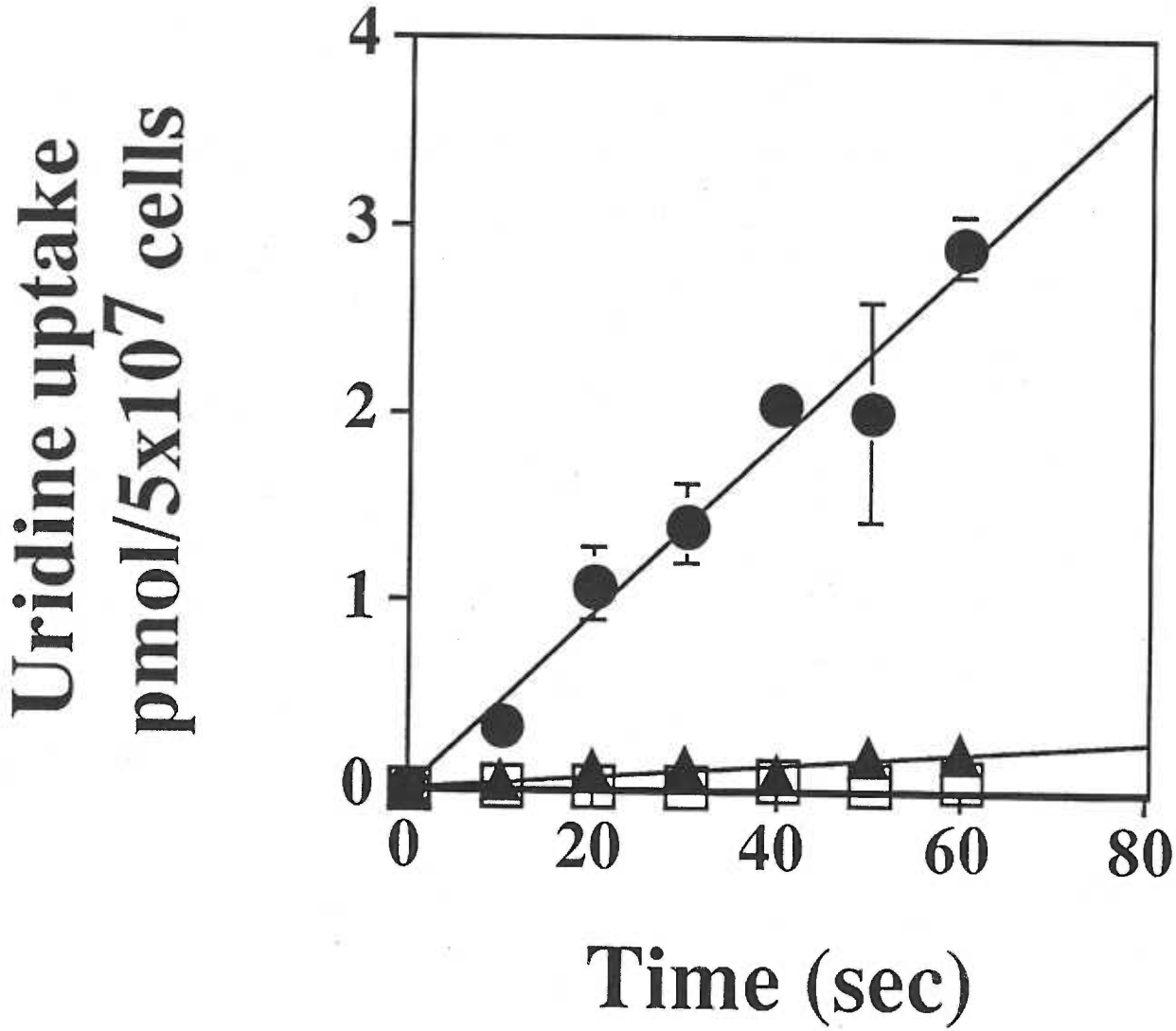

B
G183D

C337X
C

FIG. 3-4. Characterization of G183A-mediated uridine uptake.

(A) TUBA5 parasites expressing WT (●), G183A (▲) or vector pX63Hyg (□) were assayed for 1 μM [^{3}H]uridine uptake. Results are expressed as mean ± S.D. (n = 2). (B) Uptake of [^{3}H]adenosine (250 nM) by TUBA5 parasites expressing WT or G183A transporters was determined over a 10 sec period in the absence or presence of uridine (40 μM). Results are expressed as percent of uptake in the absence of uridine and each value is the mean ± S.D. (n = 3).

A



B

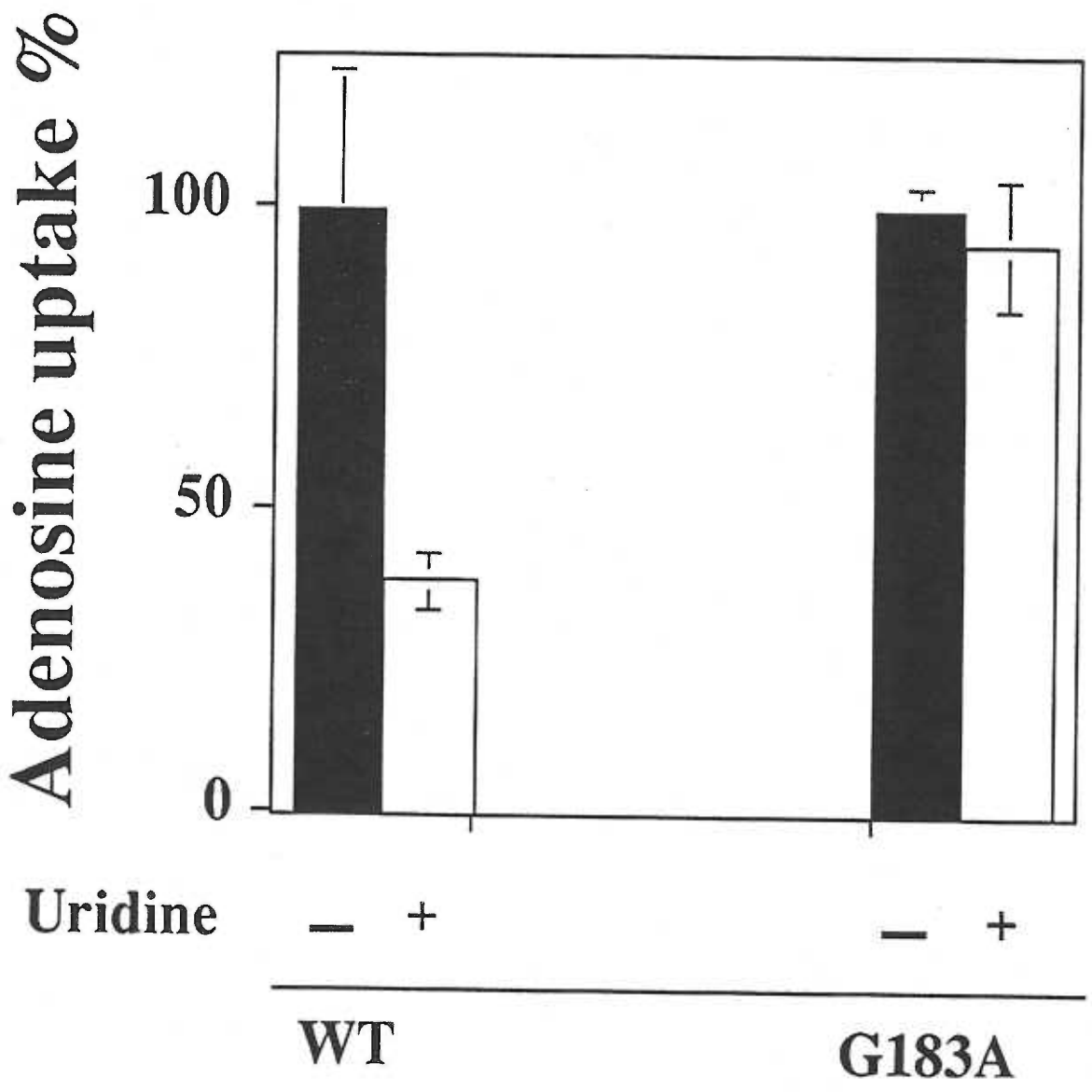


FIG. 3-5. Helical wheel representation of TMs 5 and 7 of LdNT1.1.

Residues of TM 5 and TM 7 are shown on a helical wheel of 3.6 residues per turn. Amino acids with polar side chains are indicated within squares. The positions of the G183D (G→D) and C337Y (C→Y) mutations in TMs 5 and 7 respectively are also indicated.

TM5
G→D
L
I
M
L
G
V
I
G
S
M
K
Q
V
L
G
S
M
T
V

TM7
Y→C
L
A
G
I
G
F
F
I
T
N
F
F
V
A
P
V
V
V
A
F
V
F

Table 3-1. Kinetic parameters for wild type and mutant transporters.

Substrate saturation curves for adenosine were obtained for TUBA5 parasites expressing GFP-WT, GFP-G183D and GFP-C337Y as described in the methods section. Each value is the mean ± S.D. of two independent determinations. V^n_{max} values are V_{max} values normalized to protein levels determined by Western blot analysis as described in the text.

Table 3-1. Kinetic parameters for wild type and mutant transporters.

	K_m (μM)	V_{max} (pmol/10^8 cells/sec)	V^n_{max} (pmol/10^8 cells/sec)
WT	**0.38 ± 0.02**	**3.24 ± 0.22**	**3.24 ± 0.22**
G183D	**2.3 ± 0.45**	**0.09 ± 0.008**	**0.18 ± 0.006**
C337Y	**1.99 ± 0.3**	**0.22 ± 0.014**	**0.14 ± 0.008**

Chapter 4:

Cysteine-less LdNT1.1 is a Functional Adenosine Transporter

Introduction

In chapter 3, the identification of G183 as a determinant of substrate selectivity in LdNT1.1 was described. G183 is located within the hydrophilic face of TM 5, suggesting that TM 5 could line the substrate permeation pathway through LdNT1.1.

Pore lining domains of various channel proteins including the cystic fibrosis transmembrane conductance regulator (172) and the Ca^{2+} (173) channel as well as residues comprising the substrate translocation pathway in membrane carriers such as the glucose-6-phosphate transporter (UhpT) from *Escherichia coli* (167), the yeast mitochondrial citrate transport protein (174) and the human glucose transporter GLUT1 (175) have been identified by the substituted cysteine accessibility method (SCAM). In this method, consecutive residues in a putative "channel"-lining transmembrane domain are individually replaced with cysteine residues and the susceptibility of each of these cysteine residues to chemical modification by membrane-impermeable thiol-specific reagents determined. The irreversible alteration of the functional properties of any cysteine substitution mutant by the sulfhydryl reagent is evidence that the corresponding residue in the wild type transporter is water accessible. Moreover, if approximately one half of the TM domain is water accessible with the pattern of accessibility showing a periodicity of 4, it is strong evidence that the TM domain is an α-helix with the hydrophilic face lining the aqueous translocation pathway. Implicit in such an approach is the assumption that only the water accessible surface participates in the formation of the translocation pathway and that the covalent modification of the cysteine residue interferes with transport.

The first step in analyzing TM 5 of LdNT1.1 by the SCAM is the creation of a mutant LdNT1.1 protein from which all native cysteines have been removed. Furthermore, the cysteine-less mutant must retain sufficient transport activity in order for subsequent structure/function studies to be possible. A cysteine-less mutant of LdNT1.1 in which all 5 cysteine residues have been replaced by alanines has been created and the mutated gene expressed in *Xenopus* oocytes. This cysteine-less LdNT1.1 transporter retains a significant proportion of the wild type transport activity and can therefore be used to probe the structure and function of TM 5.

Results and Discussion

Site-directed mutagenesis of C337. The relative positions of the five cysteine residues in LdNT1.1 are indicated in Figure 4-1. As previously described, cysteine-337 located in TM 7 is mutated to tyrosine in TUBA5 parasites and this mutation contributes to the transport-deficient and drug-resistant phenotype of the mutant cell line. To determine whether C337 was essential for LdNT1.1 function, site-directed mutagenesis (materials and methods, chapter 3) was used to replace this residue with alanine, serine or phenylalanine. Constructs encoding the mutated transporters were transfected into the TUBA5 parasites and tested for their ability to restore [^{3}H]adenosine transport. TUBA5 parasites expressing the C337F transporter showed only background levels of transport, indicating that amino acids with bulky side-chains were not tolerated at this position (Figure 4-2). In contrast, both C337A and C337S were clearly functional, although C337S-mediated transport occurred at a lower rate. These results indicated that C337 *per se* was not essential for adenosine transport.

Construction and preliminary characterization of the cysteine-less (C-) mutant. Starting with the construct encoding the C337A mutation, each of the remaining cysteine residues was replaced with alanine by site-directed mutagenesis. The presence of these mutations was confirmed both by diagnostic restriction digests and by sequencing and the mutated gene was cloned into the *Xenopus* expression vector, pL2-5 (materials and method, chapter 2). Both wild type and C- cRNAs were transcribed *in vitro*, injected into *Xenopus* oocytes, expressed for 3 days and assayed for uptake of 1 μM [^{3}H]adenosine (materials and methods, chapter 2). As shown in Figure

4-3, the cysteine-less LdNT1.1 transporter retained almost wild type levels of adenosine transport capability. These results indicated that none of the cysteine residues in LdNT1.1 were critical for function. As the cysteine-less mutant retains almost full transport capability, this mutant can be used to determine whether TM 5 lines the permeation pathway through LdNT1.1.

FIG. 4-1. Location of cysteine residues in LdNT1.1.

Predicted topology of LdNT1.1 indicating the positions of the 5 cysteine residues.

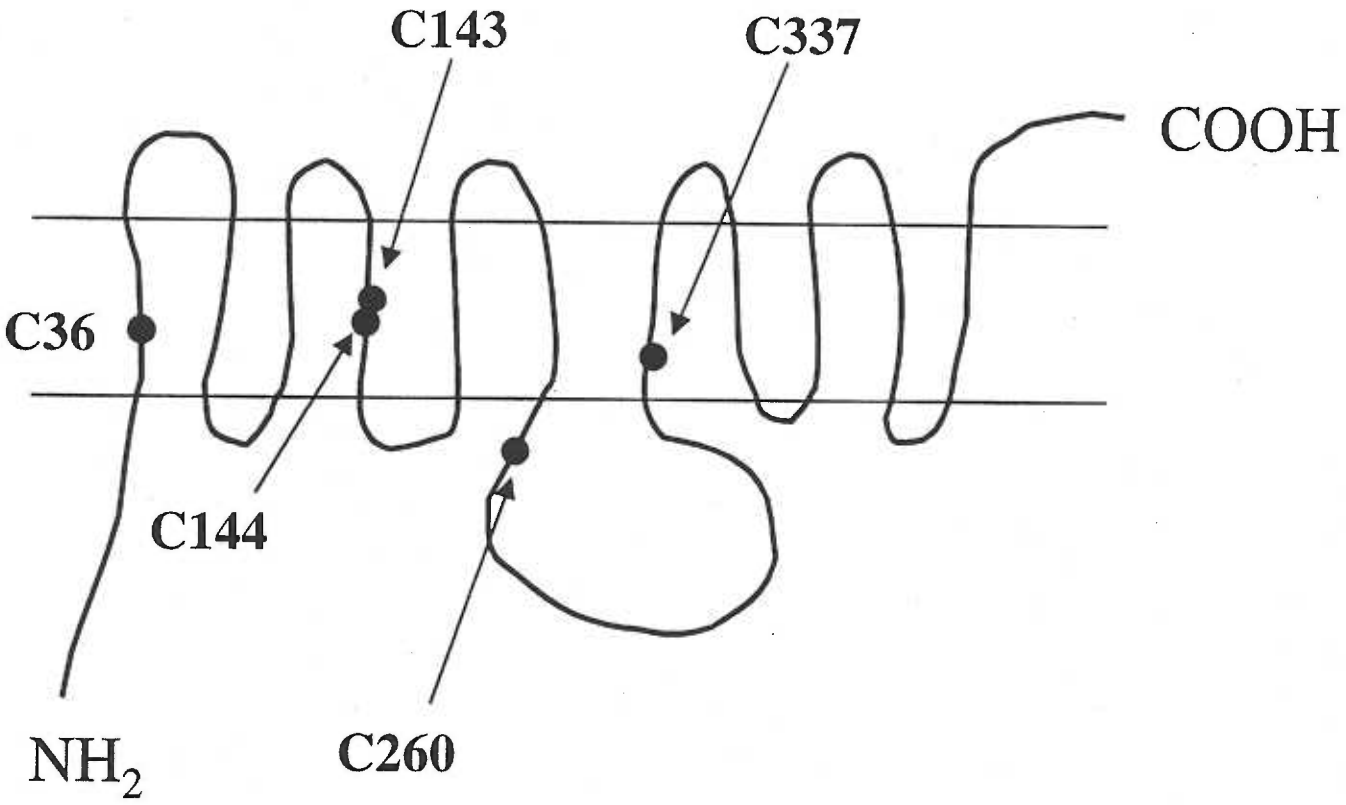

C143
C337
COOH
C36
C144
C260
NH2

FIG. 4-2. Adenosine transport by C337 mutants.

TUBA5 parasites expressing C337A (▼), C337S (●), C337Y (◊), C337F (Δ) or vector pX63Hyg (□) were assayed for 1μM [^{3}H]adenosine uptake. Results are expressed as mean ± S.D. (n = 2).

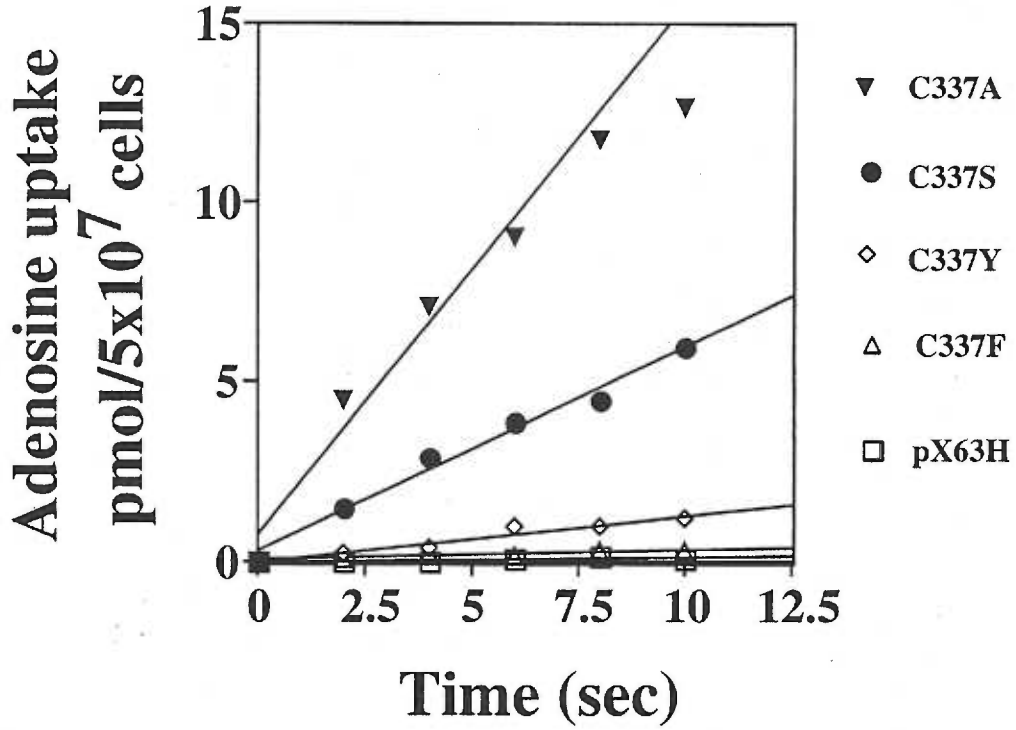
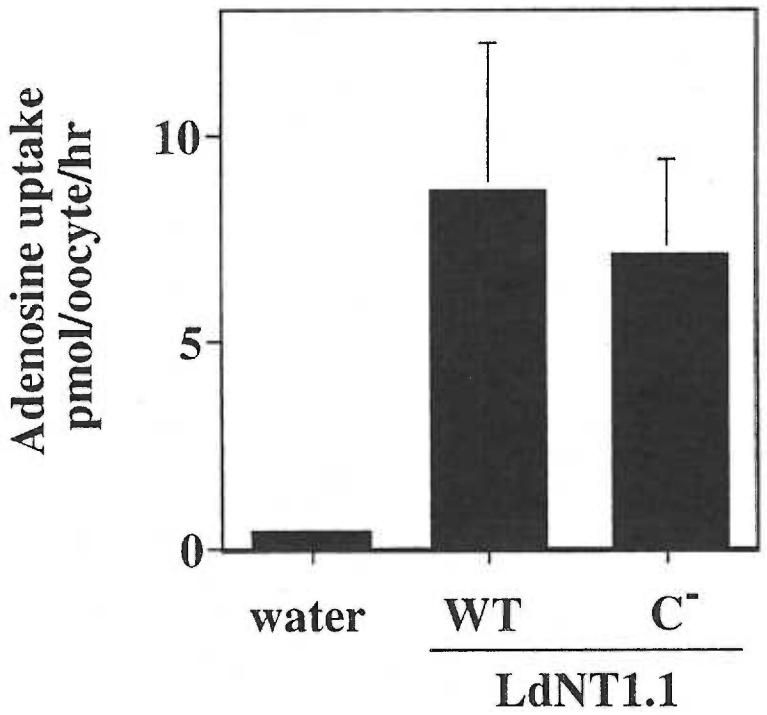

Adenosine uptake
pmol/5x10^7 cells
15
10
5
0
0
2.5
5
7.5
10
12.5
Time (sec)
C337A
C337S
C337Y
C337F
pX63H

FIG. 4-3. Functional characterization of C-/LdNT1.1 in *Xenopus* oocytes.

Xenopus oocytes were microinjected with water or ~20 ng of WT or C- LdNT1.1 cRNA, incubated at 15°C for 3 days and assayed for uptake of 1 μM [^{3}H]adenosine over 1 h (mean ± SD, n=6).


Adenosine uptake
pmol/oocyte/hr
10
5
0
water
WT
C-
LdNT1.1

Chapter 5: Discussion

This thesis describes the functional cloning of the adenosine-pyrimidine nucleoside transporter genes from *L. donovani*, the biochemical characterization of these transporters and the first steps towards understanding structure-function relationships in parasite nucleoside transporters.

The *LdNT1.1* and *LdNT1.2* genes were cloned by virtue of their ability to complement the transport-deficient and tubercidin-resistant phenotype of the mutant TUBA5 cell line. Such a functional rescue strategy can be successfully used to isolate any gene for which a genetic selection or screen can be devised. *LdNT1.1* and *LdNT1.2* are almost identical within their coding regions but very divergent in the 5' and 3' untranslated regions (UTRs). Moreover, while *LdNT1.1* is robustly expressed in promastigotes, *LdNT1.2* transcripts are undetectable in this life cycle stage of the parasite. In *Leishmania*, gene expression is usually regulated post-transcriptionally and this regulation is often mediated by sequences in the 3' UTR (176). It is therefore likely that sequences within the divergent 3' UTRs are responsible for the differential expressions of *LdNT1.1* and *LdNT1.2*. *LdNT1.2* may be selectively expressed in amastigotes, the life cycle stage within the phagolysosome of the vertebrate host. Biochemical studies have detected an adenosine-pyrimidine nucleoside transporter designated T1, in *L. donovani* amastigotes (121). Whether LdNT1.2 contributes to this transport activity remains to be determined. Alternatively, *LdNT1.2* may be selectively expressed during one of the parasite developmental stages within the midgut of the sandfly.

LdNT1.1 and LdNT1.2 have similar apparent affinities for adenosine (K_ms of 0.2 μM and 0.7 μM respectively) but LdNT1.2 has a much lower affinity for uridine (K_m = 40 μM) than does LdNT1.1 (K_m = 5 μM). These two proteins differ at only 6 amino acid positions within their primary sequence. Site-directed mutagenesis of these amino acids individually and in combination should help to determine which of these 6 amino acid residues contribute to their different kinetic properties.

The LdNT1 transporters show ~ 30% identity to members of the mammalian equilibrative nucleoside transporter family. However, they differ from the mammalian transporters in terms of substrate selectivity and affinities. The mammalian equilibrative transporters mediate the uptake of a broad range of purine and pyrimidine nucleosides and display affinities that are about two orders of magnitude lower than the parasitic nucleoside transporters for their substrates (75, 76). LdNT1.1 and LdNT1.2 exhibit more restricted substrate specificities for adenosine and pyrimidine nucleosides. In this light it is interesting that LdNT1 shows no homology to the sodium dependent h/rCNT1 transporters that exhibit a similar selectivity for pyrimidine nucleosides and adenosine (91, 95).

Genes for several parasitic nucleoside transporters have been cloned and the permeases functionally characterized (126, 131, 157). Many of these transporters have different but overlapping substrate specificities. The cloning of these genes has allowed not only the biochemical characterization of individual transporters but also a comparison of their amino acid sequences with each other and with those of the mammalian nucleoside transporters. Like the mammalian equilibrative nucleoside

transporters, the majority of the parasitic transporters are predicted to contain 11 transmembrane domains with a large intracellular loop between TMs 6 and 7. The exceptions are LdNT2 and TbAT1, which are predicted to contain 9 and 10 transmembrane segments respectively (127, 128). However, all of these topologies are predictions that have not been confirmed experimentally. Multiple sequence alignments between the parasitic and mammalian transporters have identified 16 conserved residues, most of which occur in transmembrane segments (171). The fact that these residues are conserved in transporters belonging to such evolutionarily distant organisms suggests that they may play important roles in transporter structure and function. The importance of these conserved residues can now be assessed using reverse genetic approaches.

The TUBA5 cell line is deficient in adenosine and pyrimidine nucleoside transport and resistant to tubercidin. This transport-deficient phenotype results from single but distinct point mutations within the genes coding for the LdNT1.1 transporter. This cell line therefore contains two mutant *LdNT1.1* alleles, one of which encodes a transporter with a G183D mutation in predicted TM 5 and the other a transporter with a C337Y mutation in predicted TM 7. When overexpressed, both mutant proteins unlike the wild type transporter fail to restore the adenosine transport capability and tubercidin sensitivity of the TUBA5 cells, confirming that these mutations are responsible for the transport-deficient and drug-resistant phenotype of the TUBA5 parasites. Both mutations involve substitutions with bulkier amino acids. Moreover, the G183D mutation also introduces a negative charge into a putative

transmembrane domain. Despite these large changes, the mutant proteins are produced at comparable levels to wild type and traffic correctly to the plasma membrane. Thus, the mutations produce the loss of function phenotype by impairing the ability of the transporters to function rather than affecting their synthesis or localization. In contrast, many of the mutations in the Na^+/glucose cotransporter (SGLT1) that cause glucose-galactose malabsorption impair the trafficking of the transporter to the plasma membrane (177).

It is worth noting that LdNT1.1 localizes to the plasma membrane of the cell body, but is excluded from the flagellum. A flagellar targeting signal has been identified in the isoform-1 of the *L. enrietii* glucose transporter that is both necessary and sufficient for flagellar localization (15). LdNT1.1 does not contain a flagellar targeting signal analogous to the one in isoform-1. It has been hypothesized that membrane proteins containing the flagellar targeting sequence would be sorted into the flagellar membrane whereas those that lacked this signal would perhaps by default partition into the pellicular plasma membrane surrounding the cell body. How these pellicular plasma membrane proteins are prevented from diffusing into the flagellum is still not clear although attachment to the underlying cytoskeleton may in some cases play a role (15).

Missense mutations can provide insight into structure-function relationships. While both the G183D and C337Y mutations completely abolish transport, more conservative amino acid substitutions (G183A and C337S) allow adenosine transport to occur. Therefore neither G183 nor C337 is essential for adenosine transport. Strikingly,

G183 is essential for LdNT1.1-mediated uridine transport and determines the substrate selectivity of the transporter. The G183A mutation alters substrate specificity by permitting adenosine but not uridine transport. There are several examples of single residues having dramatic effects on substrate selectivity. For example, mutation of glutamine 169 to asparagine abolished fructose transport by the *Plasmodium falciparum* hexose transporter but allowed glucose transport to occur (178). Similarly, the S318G mutation altered the selectivity of the sodium-dependent rCNT1 transporter from being pyrimidine specific to one that accepted both purine and pyrimidine nucleosides (100) and in the *Arabidopsis thaliana* metal transporter, the D103A mutation eliminated its ability to transport zinc but not iron, manganese or cadmium (179).

G183 is not among the 16 residues conserved across parasitic and mammalian nucleoside transporters. The work in this thesis however clearly demonstrates the importance of this residue in LdNT1.1 function and underscores the powerfulness of the forward genetic approach in elucidating structure-function relationships. While reverse genetics can be used to study the roles of conserved residues, forward genetic approaches have the power to identify structurally and functionally important residues that are not conserved and therefore would not be predicted to be important. Thus, a random mutagenesis approach can be used to generate a library of *LdNT1.1* mutations. Each mutated gene construct can be transfected into the TUBA5 cell line and tested for its ability to confer tubercidin resistance. Further analysis of these loss of function mutations should provide a wealth of information on residues critical for determining transporter structure or for function.

Tubercidin has not been used in experimental chemotherapy because it is extremely toxic to mammals causing hepatic and renal damage (180). However, the co-administration of tubercidin with NBMPR-P (the prodrug of NBMPR, a potent inhibitor of mammalian equilibrative but not parasitic nucleoside transporters) can restrict the toxicity of tubercidin to the parasite by preventing its uptake by mammalian cells (180). This combination therapy has been demonstrated to be effective in the treatment of schistosomiasis and trypanosomiasis in mice (180). Conceivably, tubercidin or some other toxic purine nucleoside analog could be used in conjunction with NBMPR to treat *Leishmania* infections in humans. It is therefore significant that resistance to tubercidin can result from mutations in the adenosine-pyrimidine nucleoside transporter. Mutations within *TbAT1*, the gene encoding the P2 type adenosine transporter in *T. brucei* have previously been shown to confer resistance to melaminophenyl arsenicals such as melarsen oxide (127). Since drug resistance is an increasing problem in the treatment of parasitic infections, an understanding of the mechanisms underlying drug-resistance can be very helpful in devising alternate treatment strategies.

Future directions

Although nucleoside transporters have been extensively characterized biochemically, little is known about the molecular mechanisms of transport. With the cloning of a number of nucleoside transporter genes, residues and domains that are important for transport are finally being identified. This thesis has identified G183 within TM 5 of LdNT1.1 as a critical determinant of substrate selectivity. Since TM 5 is

amphipathic, it could line the substrate permeation pathway through LdNT1.1. It should be possible to test this hypothesis with chemical modification approaches such as the substituted cysteine accessibility method (SCAM) (166). The translocation pathway through a membrane carrier has been postulated to contain three distinct domains (167). One domain comprises residues that are only exposed to the outside of the cell and the second domain comprises residues exposed only to the inside of the cell. The third centrally located domain contains residues that are alternately exposed to both the outside and the inside during the course of each transporter cycle. These domains have in fact been identified in the bacterial glucose-6-phosphate transporter UhpT (167). The identification of these domains in TM 5 would provide strong evidence that it lines the permeation pathway through LdNT1.1. The availability of a functional cysteine-less LdNT1.1 transporter will allow these studies to be performed. There are 5 cysteine residues in LdNT1.1. The creation of single cysteine mutants, each containing one of these cysteine residues should allow us to determine whether any of these native cysteines is water accessible and therefore whether the transmembrane domains in which they are located also contribute to the substrate permeation pathway.

It will also be interesting to clone and characterize the amastigote specific purine nucleoside transporter, T2 (121). Since this transporter is expressed in the life cycle stage that is infectious to humans, understanding its structure and function will be important from a pharmacological point of view. The T2 transporter gene cannot be detected in southern blots of *L. donovani* genomic DNA probed with *LdNT1.1*,

indicating that these genes are not highly homologous. Since amastigote-specific genes may not be expressed in promastigotes, it could be difficult to clone the T2 genes by their ability to complement the transport-deficient phenotype of the TUBA5 cells. It should however, be possible to functionally clone the gene or genes encoding the T2 transporter in the ade2 yeast mutants that are defective in purine biogenesis. This strategy was used successfully by Mäser *et al.* to clone the *TbAT1* gene from *T. brucei* (127). A comparison of the amino acid sequences of T2, LdNT1 and LdNT2 should provide useful information on the domains involved in determining substrate recognition.

Another area of interest is to understand the molecular mechanisms involved in the regulation of LdNT1 activity in response to changes in extracellular adenosine concentrations. Previous studies have shown that adenosine transport by LdNT1 increases almost 10-fold in response to adenosine depletion from the medium (124). In *Crithidia,* an increase in purine nucleoside transport activity under purine starved conditions is not accompanied by an increase in transporter mRNA levels (125). It is therefore likely that regulation of LdNT1 activity occurs at the level of protein synthesis, turnover or through chemical modification of the transporter in response to extracellular adenosine concentrations.

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