CHAPTER 12

Purine and Pyrimidine Metabolism in *Leishmania*

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Abstract

Purines and pyrimidines are indispensable to all life, performing many vital functions for cells: ATP serves as the universal currency of cellular energy, cAMP and cGMP are key second messenger molecules, purine and pyrimidine nucleotides are precursors for activated forms of both carbohydrates and lipids, nucleotide derivatives of vitamins are essential cofactors in metabolic processes, and nucleoside triphosphates are the immediate precursors for DNA and RNA synthesis. Unlike their mammalian and insect hosts, *Leishmania* lack the metabolic machinery to make purine nucleotides de novo and must rely on their host for preformed purines. The obligatory nature of purine salvage offers, therefore, a plethora of potential targets for drug targeting, and the pathway has consequently been the focus of considerable scientific investigation. In contrast, *Leishmania* are prototrophic for pyrimidines and also express a small complement of pyrimidine salvage enzymes. Because the pyrimidine nucleotide biosynthetic pathways of *Leishmania* and humans are similar, pyrimidine metabolism in *Leishmania* has generally been considered less amenable to therapeutic manipulation than the purine salvage pathway. However, evidence garnered from a variety of parasitic protozoa suggests that the selective inhibition of pyrimidine biosynthetic enzymes offers a rational therapeutic paradigm. In this chapter, we present an overview of the purine and pyrimidine pathways in *Leishmania*, make comparisons to the equivalent pathways in their mammalian host, and explore how these pathways might be amenable to selective therapeutic targeting.

Nomenclature

Purines and pyrimidines exist as both monomers and polymers. As monomers, they can exist in multiple forms: (1) as free, planar, heterocyclic bases (also called nucleobases); (2) as nucleosides in which an N-glycosidic linkage exists between the one carbon of the ribose or 2'-deoxyribose sugar and either the N9 nitrogen of the purine or N1 nitrogen of the pyrimidine ring; and (3) as nucleotides in which one to three phosphate groups are covalently attached to the five carbon of the ribose ring of a nucleoside. RNA and DNA are nucleotide polymers and informational macromolecules.

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Purine Metabolism

The discovery that certain pyrazolopyrimidine nucleobases and nucleosides, analogs of naturally occurring purines, are toxic to *Leishmania*, coupled with the obligatory nature of the leishmanial purine salvage pathway, has spawned considerable interest in the purine salvage pathway as a drug target.[1-4] The purine salvage pathway was initially characterized by direct enzyme measurements in parasite lysates and by metabolic incorporation experiments into intact parasites. These studies indicated that the purine salvage pathway of *Leishmania* is complex, entwined, and functionally redundant. More recent molecular and genetic investigations, coupled with the available annotated leishmanial genome sequencing projects, have revealed new details about the purine pathway of *Leishmania* and have emphasized new targets for therapeutic manipulation of the parasite.

Purine Transport in *Leishmania*

Most of our information about the transport of purines and pyrimidines into *Leishmania* has been obtained from studies with *L. donovani* promastigotes, since this form can be easily cultivated in defined medium and is amenable to genetic manipulation.[5] It is clear from experiments involving the supplementation of the cultivation medium with various exogenous purine nucleobases and nucleosides that *Leishmania* are capable of transporting and utilizing any naturally occurring purine nucleobase or nucleoside, including xanthine and xanthosine,[6-9] two purines that cannot be recycled into the nucleotide pool in mammalian cells. The first step in purine salvage is the translocation of preformed purine nucleosides or nucleobases across the parasite plasma membrane. Since nucleosides and nucleobases are hydrophilic and cannot passively diffuse across the lipid bilayer of the plasma membrane, uptake requires specialized translocation proteins or transporters. Through a battery of genetic, molecular, and biochemical studies, as well as comparative in silico analyses on the available *Leishmania* genome databases, four distinct purine transport loci have been identified within the *Leishmania* genome. In *L. donovani* these are designated as *LdNT1-4*, for Leishmania donovani Nucleoside or Nucleobase Transporter. These transporters show limited homology to each other at the amino acid sequence level (~30 % identity), as well as to a family of nucleoside transporters in mammalian cells, termed the Equilibrative Nucleoside Transporters (ENTs, Family 2.A.57 in the Transporter Classification Database, http://www.tcdb.org).[8,9] ENT transporters are distinguished by an overall similarity in predicted topology (11 transmembrane domains and a long intracellular loop between transmembrane segments 6 and 7) and possess a number of conserved or signature residues, located primarily within predicted transmembrane (TM) domains[8] (Fig. 1).

The other class of nucleoside transporter expressed in specialized mammalian cells, designated Concentrative Nucleoside Transporter (CNT) because nucleoside uptake is coupled with the cell's electrochemical gradient[9] (Family 2.A.41 in the Transporter Classification Database, http://www.tcdb.org), appears to be absent in *Leishmania* parasites based upon bioinformatic analyses of the available genome databases.

The functional expression of *Leishmania* nucleoside/nucleobase (*NT*) genes within heterologous expression systems,[13-15] as well as in nucleoside transport-deficient *L. donovani*,[7] have enabled their ligand specificities and kinetic profiles to be assigned. The *LdNT1* locus, which comprises two genes *LdNT1.1* and *LdNT1.2*, and the *LdNT2* gene encode for high affinity nucleoside transporters with nonoverlapping ligand specificities.[13,14] LdNT1 is specific for the purine nucleoside adenosine, as well as for the pyrimidine nucleosides uridine, thymidine and cytidine,[13] while LdNT2 exclusively recognizes the 6-oxopurine nucleosides inosine, guanosine and xanthosine.[7,14] In contrast, *LdNT3* and *LdNT4* appear to encode nucleobase transporters. This observation is surmised largely from parallel work on the related kinetoplastid *L. major*. Studies on LmaNT3, the *L. major* homolog of LdNT3, indicates that it transports the purine nucleobases adenine, hypoxanthine, guanine and xanthine with relatively high affinity.[15] LdNT3, which has not been characterized in detail, mediates the translocation of adenine, hypoxanthine, guanine, and xanthine in yeast (Galazka, Gessford,

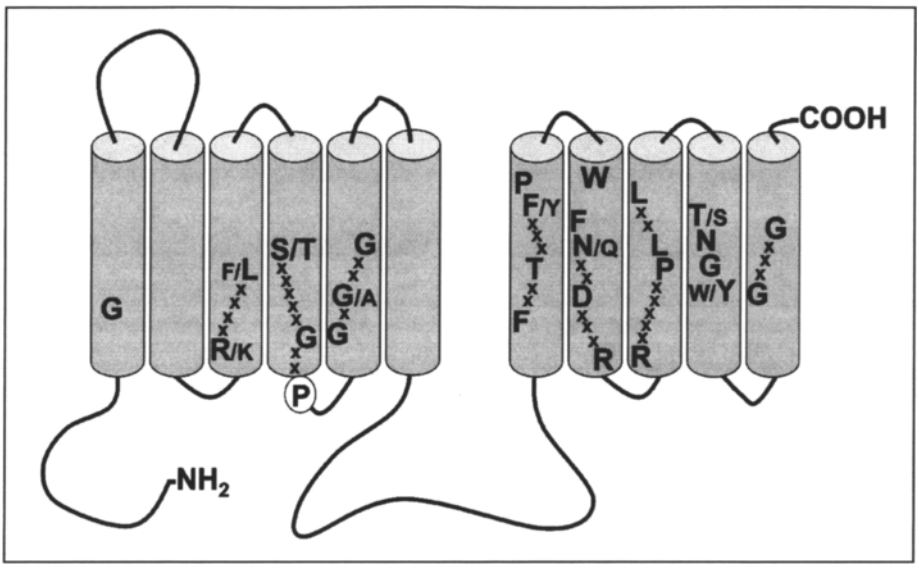

Figure 1. Signature residues delineating the equilibrative nucleoside transporter family. Sequence comparisons were made between the following ENT family members, hENT1,[10] hENT2,[11] hENT3,[12] hENT4,[12] LdNT1,[13] LmaNT1 (LmjF15.1240), LdNT2,[14] LmaNT2 (LmjF36.1940), LdNT3, LmaNT3,[15] LdNT4, LmaNT4 (LmjF11.0550), TbAT1,[16] TbNT2,[17] PfNT1,[18] and TgAT.[19]

and Ullman, unpublished). Likewise, studies on LmaNT4, the *L. major* homolog of LdNT4, suggest that this ENT is an adenine transporter, but exhibits a lower affinity for the nucleobase than LmaNT3 (Ortiz and Landfear, unpublished).

Although the leishmanial NTs share overlapping ligand specificities with mammalian ENTs, there are some notable differences. Electrophysiological measurements in *Xenopus* oocytes with heterologously expressed LdNT1 and LdNT2 demonstrate that unlike their mammalian counterparts, which are equilibrative, these transporters couple a proton for each molecule of ligand translocated.[20] Moreover, unlike most human transporters, which are exquisitely sensitive to the effects of the nucleoside transport inhibitors dipyridamole, dilazep and *S*-nitrobenzyl-4-thioinosine, the leishmanial NTs are largely refractory. The electrogenic nature of the leishmanial permeases and their refractoriness to classical mammalian nucleoside transport inhibitors suggest that the nucleoside translocation mechanism may be discrepant between *Leishmania* and human NTs.

Purine Transporters as Drug Targets in *Leishmania*

Because of the indispensable nature of purine salvage to *Leishmania,* purine transporters may serve as drug targets or as portals through which to introduce toxins. To assess the physiological consequences of loss of nucleoside transport on intact parasites and therefore to establish their suitability as drug targets, a series of null mutants have been created using targeted gene replacement and selection for loss-of-heterozygosity.[7] Thus, we have created *Δldnt1*, *Δldnt2*, and *Δldnt1/Δldnt2* knockout cell lines. Transport studies on promastigotes and amastigotes of these null mutants demonstrated an absence of adenosine transport in *Δldnt1* parasites, a lack of inosine, guanosine, or xanthosine uptake in *Δldnt2* cells, and the complete loss of purine nucleoside uptake in *Δldnt1/Δldnt2* mutants.[7] Thus, LdNT1 and LdNT2 appear to be the sole routes for nucleoside uptake in both lifecycle stages of *L. donovani.* Despite these genetic lesions *Δldnt1*, *Δldnt2*, and *Δldnt1/Δldnt2* cells are all able to grow on any single, natural purine, with the exception that parasites harboring a *Δldnt2* lesion cannot utilize xanthosine.[7] More

than likely, these discrepancies between transport and growth phenotypes can be ascribed to the extracellular metabolism of purine nucleosides to their respective nucleobases by hydrolytic activities present either in the leishmanial growth medium or on the parasite cell surface. Interestingly, a nonspecific nucleoside hydrolase that recognizes all purine nucleosides appears to localize to the parasite plasma membrane in *L. donovani*[21] and thus may provide a route for purine nucleosides to be converted to nucleobase, ligands for LdNT3 or LdNT4, thereby circumventing the *Δldnt1*, *Δldnt2*, and *Δldnt1/Δldnt2* lesions.

In spite of their lack of nucleoside transport, *Δldnt1*, *Δldnt2*, and *Δldnt1/Δldnt2* cells are as infectious and viable in J774 murine macrophages as wild type parasites during short-term infectivity studies.[7] This is unsurprising given the propensity of these mutants to grow on almost any natural purine. Consequently, our current hypothesis is that pharmacological inhibition of LdNT1 and/or LdNT2 activity is unlikely to cause a loss of viability of *Leishmania* parasites in vivo. However, these transporters may still prove effective for the specific targeting of antileishmanial drugs. Indeed, studies with *Δldnt1* and *Δldnt2* parasites have confirmed that LdNT1 and LdNT2 serve as the primary conduit for uptake of the toxic nucleoside drugs tubercidin, a toxic adenosine analog, and formycin B, a cytotoxic inosine isomer, since *Δldnt1* and *Δldnt2* mutants are highly resistant to these agents.[7]

Structure-Function Studies on Purine Transporters

The successful exploitation of nucleoside transporters in *Leishmania* as a delivery system for toxins is contingent on knowledge of the constraints governing ligand translocation, which are, thus far, largely unknown. However, preliminary structure-function studies of LdNT1 and LdNT2 have yielded some hints about which helices line the ligand translocation channel and have even uncovered particular residues that appear to play a role in ligand discrimination.

Site-directed mutagenesis of charged residues within predicted TM domains in LdNT1 and LdNT2 has revealed roles for some of these residues in transporter function. Mutation of the charged residues Glu94 in TM2 of LdNT1[22] or Asp389 in TM8 of LdNT2[23] results in a properly localized but inactive transporter. These mutant proteins may fail to either bind or translocate ligand; discrimination between these possibilities awaits a cell-free system in which to study these transporters. Intriguingly, a K153R mutation in TM4 of LdNT1 confers inosine transport capability upon the adenosine/pyrimidine nucleoside transporter,[22] and similar changes in ligand specificity have been noted when the corresponding Lys residue in CfNT2, a close relative of LdNT2 from *Crithidia fasciculata*, is mutated (Arendt et al unpublished). Mutation of an amino acid located in a similar position in TM4 of the human transporter hENT1 also affects ligand affinity,[24] suggesting that this portion of TM4 is important in ligand discrimination by ENTs.

Selection for nucleoside transport-deficient mutants by chemical mutagenesis has also highlighted important residues in leishmanial purine transporters. In the TUBA5 cell line, which is null for adenosine/tubercidin uptake,[13] *ldnt1.1* alleles carry inactivating mutations that map to TMs 5 (G183D) and 7 (C337Y) in the mutant ldnt1 proteins.[25] Interestingly, mutation of Gly183 to Ala selectively abrogates uridine transport capability, while adenosine uptake kinetics are unaltered. These data suggest that this particular Gly residue in TM 5 may line the ligand translocation channel and that the uridine and adenosine binding sites are at least partially independent. The FBD5 cell line cannot take up inosine or guanosine[14] and carries a S189L mutation in one of its mutant *ldnt2* alleles, which impairs both activity and efficient cell surface localization of the mutant ldnt2 protein.[26] Mutation of Ser189 to Ala or Thr enables cell surface expression and activity.

Both ldnt1-Gly183 and ldnt2-Ser189 are located within TM5, highlighting this predicted TM domain as critical for ligand transport. Recently, TM5 of LdNT1 has been probed extensively by the substituted cysteine accessibility method.[27] The results of this study indicate that a hydrophilic face of TM5 is accessible to solvent and that most residues on this face are protected from modification in the presence of ligand, indicating that TM5 lines the ligand translocation channel. Notably, both Gly183 and Ser187 (the equivalent of Ser189 from LdNT2)

in LdNT1 are located on this hydrophilic face, suggesting that these residues directly affect the flow of ligand through the transporter. Data from chimeras between rat and human ENTs also indicate that TMs 3-6 are important for ligand discrimination,[28,29] suggesting that similar parts of human and protozoan transporters contact ligand.

A structural model has recently been proposed for LdNT2[30] based on threading of ENT primary sequences onto the structure of the glycerol-3-phosphate transporter, a member of the major facilitator superfamily, a family with similar secondary structures to ENTs. In this model, TMs 1, 4, 5, 8, 10 and 11 of LdNT2 line a central channel (Fig. 2). Support for this model comes from the fact that nearly all of the residues found to affect transport activity are located in TMs 4, 5 and 8, all of which line the predicted pore of the ENT. This three-dimensional structural model is therefore consistent with existing mutagenesis results and makes predictions concerning the topological arrangement of helices within the protein that can be experimentally validated, potentially providing valuable structural information for the ENT family.

Another consideration for the therapeutic exploitation of purine transporters is which moieties on the ligand influence its ability to be transported first by mammalian purine transporters and then by the parasite transporters on the surface of the intracellular amastigote. This information will provide constraints for the rational design of effective purine analogs that can gain access to enzymes within the parasite. Little is known about how *Leishmania* and human nucleoside transporters contact ligand; however, the selectivities of human ENT1 vs. LdNT1/LdNT2 are suggestive. The ubiquitously expressed hENT1 transports all natural purine and pyrimidine nucleosides with moderate affinity[31,32], suggesting that key contacts are likely made to substituents common to all ligands, e.g., the ribose hydroxyls and ring nitrogens within the nucleobase moiety. Indeed, the 3' hydroxyl of ribose and N3 of the pyrimidine heterocycle (similar to N1 of the purine ring) are important for inhibition of uridine uptake by hENT1.[33] In contrast to the broad specificity of hENT1, LdNT1 and LdNT2 discriminate between amino and oxo substituents at the C6 position of the purine ring, implicating this position as particularly vital in forming protein-ligand contacts. Taken together, these observations intimate that drugs that can establish appropriate hydrogen bonds at spatial positions equivalent to N1 and C6 (oxo or amino) in the purine and 3' OH in the ribose ring may be effectively transported into the macrophage and then into *Leishmania* amastigote. In addition, structure/

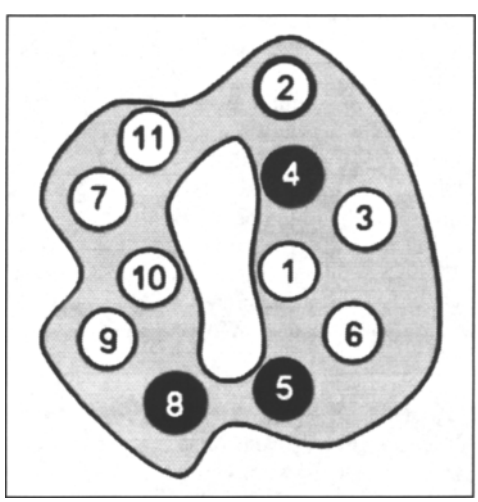

Figure 2. Structural model for arrangement of transmembrane helices in ENT family members. Residues that impact ligand discrimination and transport activity have been identified in helices 2, 4, 5, and 8, three of which are purported to line the water-filled channel (black).

activity studies of TbAT1, an adenosine/adenine transporter from *Trypanosoma brucei*, show that transported drugs need not structurally resemble purine as long as chemically equivalent contacts are provided.[34] Hence, leishmanial nucleoside transporters may provide a gateway for the uptake of diverse cytotoxins.

Purine Salvage Enzymes of *Leishmania*

Leishmania have developed an extensive network of salvage enzymes that enable them to interconvert and metabolize any host purine to the nucleotide level. Through a variety of molecular and biochemical studies, the following purine salvage pathway has been proposed in *Leishmania*.

The pathway is both complex and redundant; host purines can be metabolized to multiple products before proceeding to the nucleotide level. For example, adenosine can either be phosphorylated to produce AMP or hydrolyzed to adenine; adenine can be phosphoribosylated to form AMP or deaminated to hypoxanthine and subsequently phosphoribosylated to IMP.[2] Previous studies on either intact parasites or crude cell lysates implicate adenine phosphoribosyltransferase (APRT), hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and adenosine kinase (AK), as key enzymes within the leishmanial pathway, with the majority of flux proceeding through HGPRT.[2] These studies are complicated, however, by overlapping substrate-specificities of enzymes and the rapid rate of flux through the pathway, which makes the detection of metabolic intermediates difficult. Thus, to examine the role of each of these enzymes a series of mutants were created in *L. donovani*. All of these mutants, including a triple mutant that lacked AK, APRT, and HGPRT activity (*Δhgprt/Δaprt/ak*$^-$), were able to proliferate in defined medium containing any natural purine nucleobase or nucleoside.[35,36] This suggests that *L. donovani* in the absence of these activities are able to funnel purine nucleobases through another enzyme in the pathway, xanthine phosphoribosyltransferase (XPRT), and moreover, that none of these activities is essential for cell viability.

Introduction of the *Δxprt* lesion into wild-type cells demonstrated that XPRT is the sole route of xanthine metabolism.[6] However, all attempts to introduce the *Δxprt* mutation into cell lines containing a *Δhgprt* lesion were unsuccessful, even when high levels of exogenous adenine and adenosine were supplied, which are substrates of APRT and AK, respectively.[37] The *Δxprt* mutation could, however, be introduced readily in the presence of *Δaprt* and *ak*$^-$ mutations (*ak*$^-$*/Δaprt/Δxprt*).[37] These data suggest that all exogenous purines are distilled to substrates for HGPRT or XPRT, and that either HGPRT or XPRT is necessary for the synthesis of nucleotides and thus, for the survival of *L. donovani*.

The inability to create a cell line containing a *Δhgprt/Δxprt* lesion is due to the rapid deamination of adenine to hypoxanthine by the enzyme adenine aminohydrolase (AAH), generating a metabolic dead-end product, which cannot be utilized by the *Δhgprt/Δxprt* cell line. Leishmanial AAH is biochemically distinct from mammalian adenosine deaminase (ADA), although both catalyze a similar reaction that can be pharmacologically inhibited by 2'-deoxycoformycin (dCF).[38,39] Hence we have demonstrated that the conditional lethality of the *Δhgprt/Δxprt* mutation can only be overcome by maintaining these parasites in medium containing both adenine and dCF.[37] Not surprisingly, these mutants are unable to proliferate within murine macrophages in vitro. Currently our laboratory is investigating this *Δhgprt/Δxprt* mutant as a live vaccine candidate.

Purine Salvage Enzymes as Drug Targets in *Leishmania*

The obligatory nature of purine salvage for *Leishmania* highlights its therapeutic potential. However, targeting *Leishmania* purine salvage is complicated since humans and *Leishmania* share several of the same purine metabolizing activities, making the design of *Leishmania*-specific drugs difficult.[4] Any therapeutic strategy that exploits *Leishmania* purine salvage must target either an activity that is unique or essential to the parasite. Through genetic and biochemical analyses we have identified enzymes that are unique to *Leishmania* including XPRT, AAH and

possibly various nucleoside hydrolase activities.[6,40] Although none of these enzymes is likely to be essential, they might be exploited to selectively metabolize cytotoxic purine analogs.

Another approach is to inhibit essential, not necessarily unique, enzymes within the *Leishmania* purine salvage pathway. The genetic demonstration that HGPRT and XPRT are essential for purine salvage is a promising lead in the design of novel therapeutics. Even though humans also have HGPRT, differences may exist in the substrate-binding pockets of these enzymes wherein an inhibitor may bind the leishmanial enzyme with higher affinity.[4] This structure-based inhibitor design would be greatly facilitated by high-resolution crystal structures of *Leishmania* HGPRT and XPRT. In addition, since XPRT is also capable of transforming hypoxanthine to the nucleotide level,[6] one might imagine that a single inhibitor could cripple both HGPRT and XPRT. Furthermore, downstream enzymes such as adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ASL) (Fig. 3) may also be essential to purine salvage in *Leishmania* and are currently under investigation in our laboratory.

Alternatively, a leishmanial enzyme that also has a human counterpart may be used to selectively metabolize a subversive substrate into a toxic product. This approach is contingent upon

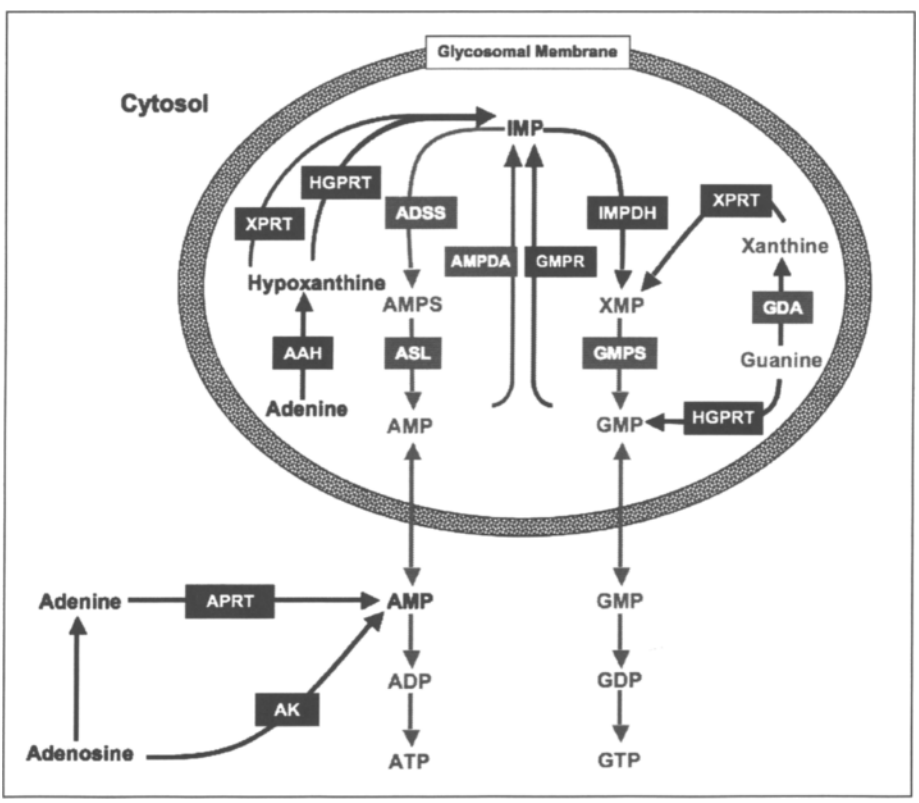

Figure 3. Compartmentalization of the purine salvage pathway of *Leishmania*. Abbreviations are as follows: AAH, adenine aminohydrolase; XPRT, xanthine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthase; GDA, guanine deaminase; AMPDA, adenosine monophosphate deaminase; GMPR, guanosine monophosphate reductase; APRT, adenine phosphoribosyltransferase; AK, adenosine kinase. Enzymes that have been localized are shown in black and those that are predicted to be in the denoted locations are depicted in gray.

structural differences in the substrate-binding pocket. Examples of subversive substrates include the pyrazolopyrimidine analogs of hypoxanthine and inosine such as allopurinol, allopurinol riboside, 4-thiopurinol, 4-thiopurinol riboside, and formycin B, which exert toxicity through incorporation into mRNA.[3,41] *Leishmania* are capable of efficiently metabolizing all five of these pyrazolopyrimidines into nucleotides, whereas humans are not.[41] By far the most studied pyrazolopyrimidine is allopurinol. The clinical efficacy of allopurinol, however, has been disappointing, perhaps as a consequence of competition by natural substrates like hypoxanthine with its metabolism.[4] Nevertheless, the tactic of using purine analogs that are selectively metabolized by the enzymatic machinery of *Leishmania* remains appealing.

The Compartmentalization of Purine Salvage in *Leishmania*

The subcellular milieu of a protein is an important determinant of its specialized function and can also impact drug-targeting paradigms. In *Leishmania* and related parasites, some of the major purine salvage enzymes are compartmentalized within the glycosome, a fuel metabolizing microbody that is unique to these parasites.[42] Glycosomal targeting can be mediated by either a COOH-terminal tripeptide or by a degenerate NH_2-terminal signal designated peroxisomal targeting signal-1 (PTS-1) or peroxisomal targeting signal-2 (PTS-2), respectively.[43] It is not known whether proteins lacking these signals can also gain access to the glycosome by "piggy-backing" on proteins with bonafide targeting signals. By direct confirmation of location by immunocytochemical methods (highlighted in black in Fig. 3) or inferred from their primary sequences (highlighted in gray in Fig. 3), the purine salvage pathway is thought to be compartmentalized in *Leishmania* between the glycosome and cytosol as depicted in Figure 3[44,45] (Jardim, A. unpublished; Boitz, J. unpublished).

The fact that key purine salvage components are localized within the glycosome necessitates, perhaps, the translocation of hypoxanthine, guanine, and xanthine across this intracellular membrane. However direct fluorescence studies with episomally expressed LdNT1, LdNT2 and LdNT3, all tagged at the NH_2-terminus with green fluorescent protein, suggest that these transporters predominantly localize to the parasite plasma membrane and flagellum[23,25] (Carter et al unpublished). That low levels of these proteins, not detectable in these experiments, are located within the glycosomal membrane, cannot be excluded, however.

The basis for the compartmentalization of the purine salvage pathway within *Leishmania* is not obvious. Glycosomal location may provide a plentiful source of ribose-5-phosphate from the pentose phosphate pathway, which is also sequestered within the glycosome.[42] Ribose-5-phosphate is needed for the synthesis of phosphoribosylpyrophosphate (PRPP), a cosubstrate for all PRT reactions. However, the purine salvage mutant *Δhgprt/Δxprt* complemented with episomal constructs of *hgprt* or *xprt* that lack a PTS-1 and thus mislocalize to the parasite cytosol, grows robustly in either hypoxanthine or xanthine.[37] This suggests that the glycosomal location of these key components of the purine salvage pathway is not necessary for their function or for parasite viability. Despite the lack of an unambiguous explanation for the unusual organellar distribution of purine salvage enzymes in *Leishmania* and related trypanosomatids, the clear-cut association of therapeutically germane purine salvage enzymes within the glycosome is noteworthy not only from a biological perspective but also from a drug development point of view, as drugs that target HGPRT or XPRT, such as the pyrazolopyrimidine nucleobase analogs[3,46] must traverse the glycosomal membrane to exert their antiparasitic effects.

Pyrimidine Metabolism

Our knowledge of pyrimidine metabolism in *Leishmania* is considerably less detailed than our understanding of purine metabolism, although it has long been known that *Leishmania* is prototrophic for pyrimidines. Previous biochemical studies in combination with more recent genetic and in silico analyses have allowed the de novo biosynthetic and salvage pathways to be defined. Moreover, several lines of evidence (discussed below) suggest that pyrimidine metabolism may present a viable target for pharmacological intervention.

Pyrimidine Biosynthesis in *Leishmania*

In both *Leishmania* and humans, de novo pyrimidine synthesis involves the sequential action of six enzymes: glutamine-dependent carbamoylphosphate synthetase (CPS), aspartate carbamoyltransferase (ACT), dihydroorotase (DHO), dihydroorotate dehydrogenase (DHOD), orotate phosphoribosyltransferase (OPRT) and orotidine monophosphate decarboxylase (OMPDC)[47] (Fig. 4). Despite the obvious similarities in biochemical activities, there are striking discrepancies between the pyrimidine biosynthetic enzymes of *Leishmania* and humans with respect to their organization into multi-functional polypeptides, allosteric regulation, use of cofactors and cellular localization. In humans, a single gene encodes a multifunctional protein encompassing the first three pyrimidine biosynthetic enzymes (designated CAD for CPS-ACT-DHO),[48] while the leishmanial enzymes are encoded by separate genes (http://www.genedb.org; Yates and Ullman, unpublished). Allosteric regulation of CPS governs the rate of pyrimidine synthesis in human cells, and the enzyme is inhibited by both UTP and CTP and stimulated by PRPP.[49] In contrast, the leishmanial CPS is preferentially inhibited by UDP and is unaffected by PRPP.[50] Human DHOD (H-DHOD) is localized to mitochondria and requires ubiquinone as a cofactor,[51] while the *Leishmania* DHOD (L-DHOD) is cytoplasmic[47] and uses fumarate rather than ubiquinone as a cofactor.[52] The last two enzymes of both the mammalian and leishmanial pyrimidine pathways, OPRT and OMPDC, are fused to form a bifunctional protein. However, the arrangements of the two enzymes are reversed; mammalian cells express an OPRT-OMPDC, whereas the leishmanial enzyme is OMPDC-OPRT. Furthermore, OPRT and OMPDC are cytosolic enzymes in mammals, while the presence of a PTS-1 as well as cell fractionation studies[47,53] establish a glycosomal milieu for the parasite OMPDC-OPRT bifunctional protein. This is the only enzyme present within the pyrimidine de novo and salvage pathways that has a glycosomal location.

Another striking feature in *Leishmania spp.* and other trypanosomatids is the organization of the pyrimidine biosynthetic genes[54] (http://www.genedb.org; Yates and Ullman, unpublished). In *Leishmania* all six activities are encoded within a contiguous genomic segment of approximately 25 kilobases. This operon-like grouping of genes involved in a single metabolic pathway is remarkable in view of the fact that genes for virtually every other metabolic pathway are scattered throughout the leishmanial genome.[55]

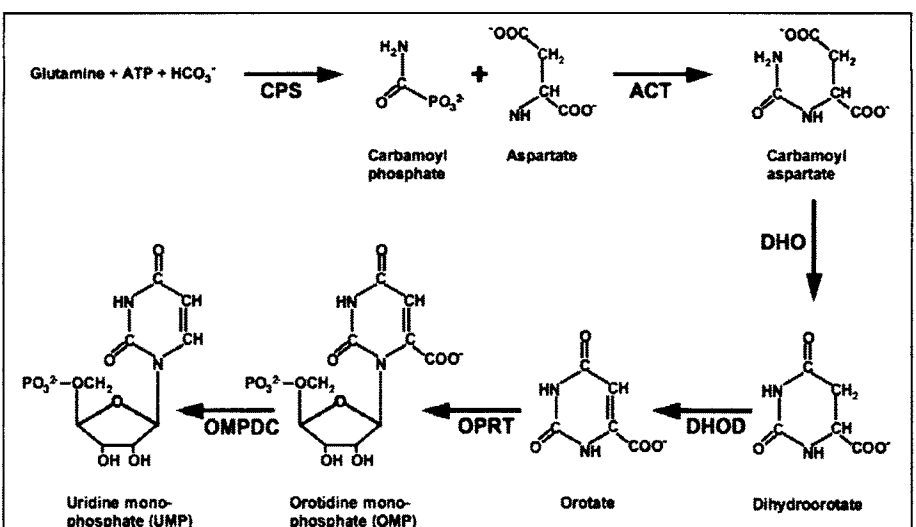

Figure 4. De novo pyrimidine biosynthesis in *Leishmania*. Abbreviations are defined in the text.

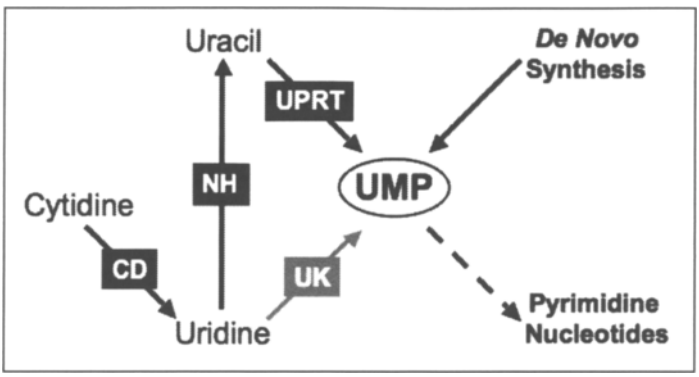

Figure 5. Pyrimidine salvage and nucleotide synthesis in *Leishmania*. Abbreviations are described in the text. Gray text box and arrow indicate that UK enzymatic activity has not been detected. The broken arrow represents the enzymatic steps for conversion of UMP into all other pyrimidine nucleotides as described in the text.

Pyrimidine Salvage and Nucleotide Synthesis in *Leishmania*

The presence of a de novo synthesis pathway implies that pyrimidine salvage may be functionally redundant and potentially less critical to the nutrition of the parasite than purine acquisition. Similar to purine salvage, pyrimidine salvage is initiated by the translocation of pyrimidine nucleosides or nucleobases across the parasite cell membrane via specific transporters. As described earlier, leishmanial NT1 is responsible for the transport of pyrimidine nucleosides.[13] The sole pyrimidine nucleobase transport activity in *Leishmania* has been characterized biochemically in *L. major* (LmU1) and shown to recognize uracil exclusively and with high affinity,[56] but has not yet been cloned.

Upon entry into the parasite, all pyrimidines (except thymidine, cytosine, thymine) are likely converted to uracil, which is phosphoribosylated to UMP by uracil phosphoribosyltransferase (UPRT; Fig. 5).

Leishmania also possess a cytidine deaminase (CD) activity that converts cytidine and deoxycytidine to uridine and deoxyuridine, respectively.[57] Uridine can then be converted to uracil by an inosine-uridine nucleoside hydrolase enzyme[58] and, possibly by other nucleoside hydrolases (NH; Yates and Ullman, unpublished). There is conflicting evidence concerning the existence of a uridine kinase (UK) activity, a more direct route for uridine salvage. UK activity was not detected in *L. mexicana*[47] and unpublished data from this laboratory imply, but do not prove, that UK is also absent in *L. donovani*. However, a gene encoding a putative UK activity is present in the *L. major* genome database. Thymidine is salvaged relatively poorly[59] and is likely directly phosphorylated to TMP by a thymidine kinase (TK), for which putative genes are present in both *L. major* and *L. infantum*.

UMP, the product of both the biosynthetic and salvage pathways, serves as the precursor for all other pyrimidine nucleotides.[60] It is converted to UDP and UTP by nucleotide kinases, UTP is aminated to CTP by CTP synthetase (CTPS), deoxyribonucleotides are synthesized from ribonucleoside diphosphates by ribonucleotide reductase (RR), and thymidylate nucleotides are produced via reductive methylation of dUMP utilizing N^5,N^{10}-methylenetetrahydrofolate as the methyl donor, a reaction catalyzed by a bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) protein.

The Pyrimidine Pathway as a Drug Target in *Leishmania*

A common strategy for drug development is to target enzymes essential for viability. While it is unlikely that pyrimidine salvage is essential, the possibility that de novo pyrimidine biosynthesis is necessary for growth and virulence of *L. donovani* was tested by creating a *Δcps* knockout via

targeted gene replacement (Yates and Ullman, unpublished). Parasites deficient in CPS were auxotrophic for pyrimidines and required supplementation with exogenous pyrimidines for survival and proliferation. Since it is not known if exogenous pyrimidines present in the phagolysosome or mammalian bloodstream are sufficient to circumvent the *Δcps* lesion, we are currently assessing the ability of *Δcps L. donovani* to infect macrophages in vitro. In the obligate intracellular parasite *Toxoplasma gondii*, which both synthesizes and salvages pyrimidines, *Δcps* mutants were avirulent and moreover, elicited a protective immune response.[61] This suggests that the levels of exogenous pyrimidines encountered by the parasite are inadequate to sustain growth. Further evidence supporting the notion that de novo pyrimidine biosynthesis is essential comes from studies of the related trypanosomatid, *Trypanosoma cruzi*, demonstrating that DHOD deficiency is lethal.[62] In *L. donovani*, the glutamine analog acivicin, which inhibits CPS and other enzymes that utilize glutamine,[63] kills both promastigote and intracellular amastigote forms.[64] Similarly, the transition state analogue N-(phosphonoacetyl)-L-aspartic acid (PALA), which specifically targets ATC, inhibits growth of both life cycle stages of *L. donovani*.[65] Taken together, these results intimate that the de novo pyrimidine biosynthesis pathway is essential for virulence and may, therefore, represent a viable therapeutic target.

To date no drugs preferentially target leishmanial components of the pyrimidine biosynthetic pathway. This is likely due to similarities in enzymatic activities shared between *Leishmania* and humans. For example, acivicin and PALA inhibit both human and leishmanial pyrimidine biosynthesis. Similarly, classical inhibitors such as methotrexate, which target leishmanial DHFR-TS, an essential component of thymidylate synthesis,[66] are poor anti-leishmanial drugs because they also inhibit the human enzyme.[67]

In contrast, the unique properties of L-DHOD make it a promising target for drug development. L-DHOD uses fumarate rather than ubiquinone as a cofactor, implying that the substrate-binding pocket differs significantly from H-DHOD. This conjecture is supported by a comparison of the crystal structure of the fumarate-utilizing *Lactococcus lactis* DHOD to that of H-DHOD.[68] That structural differences in DHOD enzymes are amenable to the discovery and design of selective inhibitors is supported by the development of specific inhibitors of other parasite DHOD enzymes, specifically inhibitors of the *P. falciparum* enzyme.[69] Likewise, structural differences in the leishmanial and mammalian bifunctional enzymes that encode OPRT and OMPDC may also be amenable to selective inhibition.

Leishmanial UPRT is a unique enzyme that, while probably not essential, could potentially be subverted to selectively metabolize toxic pyrimidine analogs. Identifying analogs selectively toxic to *Leishmania* could be challenging, as evidenced by the toxicity of the UPRT substrate 5-fluorouracil to both *Leishmania*[56] and humans.[70] While humans lack UPRT, they incorporate 5-fluorouracil into the nucleotide pool via alternative mechanisms; mechanisms that must be bypassed for any uracil analog to demonstrate selective toxicity.

Summary

Purine and pyrimidine metabolism in *Leishmania* offers several metabolic steps for therapeutic intervention. The results of recent molecular and biochemical studies have elucidated the details of the essential pathways for purine and pyrimidine acquisition/biosynthesis, brought to light significant differences between the biochemistry of leishmanial and human enzymes, and allowed the identification of "Achilles heels" in these pathways. These investigations have also uncovered several unique activities, which may be amenable to pharmacological exploitation. These include the leishmanial nucleobase and nucleoside transporters whose novel ligand specificities make them useful as portals through which to target cytotoxins, and the parasite-specific enzymes XPRT, AAH and UPRT whose activities may be plundered to activate prodrugs. Moreover, selective metabolism of prodrugs to the nucleotide level is achievable in *Leishmania* despite overlapping activities with human counterparts. Given the literally thousands of purine and pyrimidine analogs already available for high-throughput screening via cell-based assays or using recombinant enzymes, the future development of purine- or pyrimidine-based antileishmanial agents is quite promising.

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