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## Purine salvage in *Leishmania*: complex or simple by design?

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### Abstract

Purine nucleotides function in a variety of vital cellular and metabolic processes including energy production, cell signaling, synthesis of vitamin-derived cofactors and nucleic acids, and as determinants of cell fate. Unlike their mammalian and insect hosts, *Leishmania* cannot synthesize the purine ring *de novo* and are absolutely dependent upon them to meet their purine requirements. The obligatory nature of purine salvage in these parasites, therefore, offers an attractive paradigm for drug targeting, and consequently, the delineation of the pathway has been under scientific investigation for over 30 years. Here we review recent developments that reveal how purines flux in *Leishmania* and offer a potential ‘Achilles’ heel’ for future validation.

### The leishmanial purine salvage pathway revealed

The inability of *Leishmania* to synthesize the purine ring was established in 1978 by Marr, Berens and Nelson who demonstrated that this genus could not convert [<sup>14</sup>C]-formate, [<sup>14</sup>C]-glycine, or [<sup>14</sup>C]-serine purine ring precursors, into adenylate and guanylate nucleotides [1]. By contrast, the incorporation of [U-<sup>14</sup>C] glucose into purine nucleotides suggested both a capacity for purine salvage and the synthesis of phosphoribosylpyrophosphate (PRPP), a substrate for several key enzymes of purine salvage [1]. As a consequence of their absolute reliance on an external purine source, *Leishmania* have developed an extensive purine acquisition pathway that enables them to scavenge purines from their culture or host milieu, and the parasite is capable of incorporating virtually any naturally occurring purine nucleobase or nucleoside into its nucleotide pools [1–5].

The *Leishmania donovani* purine salvage pathway has been largely delineated using biochemical, molecular, and genetic tools over the past three decades [1–15]. Early metabolic flux experiments with radiolabeled purine precursors helped establish a nearly complete picture of the activities that comprise the purine salvage pathway (Figure 1). The genes for all of these purine salvage pathway components have now been identified using molecular genetics approaches or from the annotated leishmanial genomes [16–19].

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## Purine salvage and interconversion in *L. donovani*

The *L. donovani* purine salvage activities include three phosphoribosyltransferases, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), and adenine phosphoribosyltransferase (APRT), that catalyze the phosphoribosylpyrophosphate (PRPP)-dependent phosphoribosylation of purine bases [1,5], adenosine kinase (AK) that phosphorylates adenosine [20,21], and a multiplicity of purine interconversion enzymes (Glossary) [1–3]. These purine salvage components are summarized in Figure 1. *Leishmania* express several enzymes that catalyze the breakdown of host nucleosides, nucleotides, and nucleic acids prior to entry into the parasite purine pools. At least four nucleoside hydrolase enzymes have been identified in *Leishmania* [3,22–25]. IUNH, an inosine-uridine nucleoside hydrolase, also designated as the non-specific nucleoside hydrolase [23,24,26], cleaves inosine, uridine, cytidine, xanthosine, adenosine and guanosine to the corresponding base. *L. donovani* IUNH has been immunolocalized to specific foci inside the cell membrane, but this location has not been verified by other biochemical methods [23]. Of the other two nucleoside hydrolases annotated in the leishmanial genomes, one is specific for inosine and guanosine [22], and the other recognizes inosine, adenosine, and guanosine [25] (J. M. Boitz, *et al.*, unpublished). A fourth nucleoside hydrolase activity that is specific for 2'-deoxyribonucleosides has also been detected in *L. donovani*, but its gene and protein have not been identified to date [22]. *L. donovani* also express two membrane-bound 3'-nucleotidases/nucleases that are located on the external cell surface of the parasite [27–29]. These 3'-nucleotidases/nucleases either generate free nucleosides via the hydrolysis of 3'-nucleotides or hydrolyze nucleic acids to 5'-nucleotides. The 5'-nucleotides are further metabolized by membrane-bound acid phosphatases to their respective nucleosides [30–32], which are subsequently translocated into the parasite by cell surface nucleoside transporters.

## Purine transport in *L. donovani*

The first step in purine acquisition into the parasite involves the transport of purine nucleobases and nucleosides across the parasite plasma membrane. Free nucleosides and nucleobases that are scavenged from the host are translocated into the parasite by a variety of specialized transporters that have distinct specificities for particular ligands [33]. Four discrete transporters have been identified in *L. donovani*, and these have been designated LdNT1-4 for *L. donovani* Nucleoside or Nucleobase transporter [33,34]. LdNT1 is specific for adenosine and pyrimidine nucleosides [35], LdNT2 transports the 6-oxopurine nucleosides inosine, guanosine, and xanthosine [36–38], LdNT3 is a purine nucleobase transporter [39,40], and LdNT4, which is homologous to the *L. major* transporter LmaNT4 that preferentially transports purine nucleobases at acidic pH [39,41], is likely a purine nucleobase transporter. LdNT1-4 share approximately 30% identity and are topologically homologous to members of the mammalian Equilibrative Nucleoside Transporter (ENT) family [33]. LdNT1-4 also display several conserved ENT signature residues that are located within the predicted transmembrane domains of these transporters [33]. However, despite their sequence and topological similarities with their ENT counterparts in higher eukaryotes, the LdNT1-4 are electrogenic symporters, coupling a proton for each molecule of ligand transported and creating a positive inward flux across the parasite plasma membrane [33].

## Functional redundancy within the purine salvage pathway

Once transported, exogenous nucleosides and nucleobases are incorporated into the parasite adenylate and guanylate nucleotide pools via a complex and intertwined purine salvage pathway that contains several functional redundancies. For example, adenosine can either be directly phosphorylated by AK to form AMP or catabolized to adenine by at least one nucleoside hydrolase [3,22]. Adenine can be phosphoribosylated by APRT or deaminated to

hypoxanthine by adenine aminohydrolase (AAH) and subsequently phosphoribosylated by HGPRT or XPRT [2]. Inosine can be hydrolyzed by at least two nucleoside hydrolases to form hypoxanthine, which in turn is phosphoribosylated to inosine monophosphate (IMP) by HGPRT. Guanosine is cleaved to guanine, which can theoretically be phosphoribosylated by HGPRT or deaminated by guanine deaminase (GDA) to xanthine [2,12], which is converted to xanthine monophosphate (XMP) by XPRT. Xanthosine, which is probably not produced in great quantities by host cells, is catabolized to xanthine and salvaged through XPRT [2]. The intricacy of the purine pathway is further magnified by its compartmentalization between the cytosol and the glycosome, a unique metabolic organelle in these organisms [10,42–49]. The sequestration of cytosolic and glycosomal purine salvage pathway enzymes is discussed in Box 1.

### Box 1

#### Compartmentalization of the purine salvage pathway

*Leishmania*, like all members of the kinetoplastid family, harbor fuel-metabolizing organelles called glycosomes [43,46,64]. Although glycosomes contain several archetypal peroxisomal functions, such as fatty acid  $\beta$ -oxidation and ether-lipid biosynthesis, they lack the hallmark  $H_2O_2$  producing catalases that are a defining feature of peroxisomes [46]. In addition to housing the first seven steps of glycolysis, glycosomes also compartmentalize specific enzymes involved in gluconeogenesis, the pentose phosphate pathway, squalene and sterol synthesis, pyrimidine and polyamine biosynthesis, and purine salvage, although it should be noted that some glycosomal enzymes can also be found in the cytosol [46]. Most glycosomal matrix proteins contain a peroxisomal targeting signal (PTS) that directs their import into the glycosome. This may be either a C-terminal tripeptide PTS1 or a more divergent N-terminal nonapeptide PTS2. For those glycosomal matrix proteins that lack a PTS, a ‘piggybacking’ mechanism through interaction with other glycosome-destined proteins has been proposed [45]. Through bioinformatic searches for potential PTS motifs, as well as from direct experimental validation, it appears that HGPRT, XPRT, IMPDH, [47–49] GMPR (A. Jardim, *et al.* unpublished) and possibly GMPS are localized within the glycosome (Figure I). This glycosomal targeting is not essential for the function of certain enzymes, as deleting the PTS1 motifs from HGPRT, XPRT, IMPDH or GMPR, does not affect the ability of the truncated products to complement  $\Delta hgprt$ ,  $\Delta xprt$ ,  $\Delta impdh$ , [47,48,58], and  $\Delta gmpr$  cells, respectively.

Although the reasons for this dual compartmentalization are unclear, it has been postulated that the ‘tight quarters’ of the glycosome may serve to increase metabolic flux, but this theory, at least for glucose metabolism, has not been supported by the mathematical models produced by Barbara Bakker and colleagues [46]. The bulk of purine nucleotide synthesis is likely to occur within the glycosomal matrix since both HGPRT and XPRT, which catalyze the vast majority of flux through the salvage pathway, reside there. Another intriguing, but as of yet unproven, thesis that has recently been put forth for compartmentalization within the glycosome is that this organelle provides these parasites with more metabolic flexibility and an ability to adapt quickly and efficiently to changes in their host environments [46].

The regulation of purine metabolism in the glycosome may perhaps be achieved through inherent nucleotide sensing mechanisms that are harbored within IMPDH and GMPR [17]. Multiple sequence alignments show that both of these enzymes accommodate a tandemly repeated 60 amino acid cystathionine  $\beta$ -synthase (CBS) regulatory domain, an element that binds ATP, AMP, and S-adenosylmethionine [17] and has been postulated to bind GMP and GTP [49]. Enzyme kinetic studies have revealed that IMPDH activity is

not affected by ATP but is dramatically reduced by elevated levels of GMP and GTP [49]. By contrast, *in vitro* experiments showed that the GMPR activity is accelerated by increased levels of GTP, which would promote the shunting of accumulating levels of GMP into the adenylate pool. Interestingly, the GMPR CBS domain is unique in that it binds both ATP and GTP, directly governing the catalytic response to the adenylate and guanylate levels in the cell (A. Jardim, *et al.*, unpublished). Given that IMPDH and GMPR are glycosomal enzymes, it is tempting to speculate that compartmentalization of these purine interconversion pathways may be a vital mechanism for controlling purine levels in *Leishmania*.

## Genetic analyses reveal fluxes through the purine salvage pathway

The mere existence of a gene or its product cannot point to its functional relevance or its involvement within the purine salvage pathway. However, the evolution and advancement of genetic tools in *Leishmania*, such as targeted gene replacement [50,51], has enabled a systematic, and nearly complete, genetic dissection of this pathway in *L. donovani*. The generation of *Leishmania* mutants that are null for an assortment of enzymes in the purine salvage pathway has facilitated the delineation between functional and functionally redundant routes of purine acquisition and has pinpointed enzymes that might be essential for *L. donovani* survival. A selection of the *L. donovani* purine salvage mutants that are either genetically null or enzymatically dysfunctional for purine salvage or interconversion is listed in Table 2.

Mutational and gene targeting schemes in *L. donovani* have clearly established that none of the four known enzymes capable of converting host purine nucleobases or nucleosides to the nucleotide level, HGPRT, APRT, XPRT, or AK, is by itself essential and that parasites with a deficiency in any one of these enzymes can still effectively salvage most, if not all, purines, although the routes of purine metabolism are affected by these lesions [12,20]. The phenotypic characterization of the  $\Delta hgprt$ ,  $\Delta aprt$ , and  $\Delta xprt$  null mutants, in particular, has revealed some unusual and novel features about purine salvage in *Leishmania*, and most importantly, provided clear evidence for how purines are funneled through the pathway [12]. For example, although cell lysates prepared from  $\Delta hgprt$  parasites cannot incorporate [<sup>14</sup>C]hypoxanthine into purine nucleotides over a short time course, intact  $\Delta hgprt$  cells are still capable of incorporating [<sup>14</sup>C]guanine at rates similar to wild type levels and can proliferate indefinitely in guanine as a sole purine source [12]. This ability to salvage and grow in guanine can be explained by the presence of XPRT, which, in fact, plays a key but indirect role in guanine salvage by *L. donovani*. The significance of XPRT in guanine salvage is supported by the fact that  $\Delta xprt$  *L. donovani* are unable to grow in guanine, as well as xanthine and xanthosine, providing genetic proof that XPRT is perhaps the only physiologically important route by which these three purine nutrients are incorporated into the parasite nucleotide pools [12]. Even though kinetic studies have revealed that guanine is a high affinity substrate for *L. donovani* HGPRT [52], the fact that  $\Delta xprt$  parasites fail to grow in guanine indicates that guanine is deaminated to xanthine, a dead end substrate in the  $\Delta xprt$  background [12]. A robust guanine deaminating activity has been described [1,2], and a gene encoding a presumptive GDA has been annotated in the genomes of all *Leishmania* species [16,17,19]. Thus, it appears that the majority of intracellular guanine is preferentially transformed to xanthine by GDA and elevated to the nucleotide level by XPRT.

Similarly, analysis of purine metabolism by  $\Delta aprt$  *L. donovani* demonstrates that this null mutant could efficiently incorporate [<sup>14</sup>C]adenine into purine nucleotides, supporting the existence of at least one additional route for adenine metabolism in the parasite [12]. The deamination of adenine to hypoxanthine via AAH has been documented [2,15,53], and this

route of adenine flux in the  $\Delta aprt$  parasites was confirmed by the addition of 2'-deoxycoformycin (dCF), a known inhibitor of AAH, to the culture medium [12]. The addition of dCF to  $\Delta aprt$  parasites obliterated [ $^{14}\text{C}$ ]adenine incorporation into nucleotides by preventing adenine hydrolysis to hypoxanthine and subsequent phosphoribosylation of the 6-oxypurine by HGPRT. The ability of adenine to serve as the purine nutrient for  $\Delta aprt$  parasites indicates that the conversion of adenine to hypoxanthine by AAH is sufficiently robust for maintenance of purine nucleotide pools and to sustain the continuous growth of the null parasites [12]. Recent experiments have demonstrated that purified, recombinant AAH, as well as live parasites, catalyzes the robust conversion of adenine to hypoxanthine [10] and its pivotal role in funneling salvaged purines through hypoxanthine to IMP. Although AAH is homologous to the mammalian adenosine deaminase enzyme, the *L. donovani* AAH does not recognize adenosine as a substrate. Despite its central role in adenine metabolism, AAH is not essential for parasite viability, since  $\Delta aah$  parasites are capable of utilizing the full spectrum of salvageable purine nucleobases and nucleosides, implying *L. donovani* can divert adenine and adenosine metabolism through APRT and possibly AK in the absence of AAH [10].

These genetic studies in promastigotes underscore the significance of both GDA and AAH in purine interconversion and salvage by *L. donovani* and indicate that the majority of intracellular guanine and adenine is metabolized to xanthine and hypoxanthine, respectively, and then converted to the nucleotide level by XPRT and/or HGPRT. These mutational analyses also imply that the formation of nucleotides from xanthine and hypoxanthine are the primary salvage routes utilized by *L. donovani*. A depiction of the purine salvage pathway showing the relative flux and presumptive fates of metabolites is shown in Figure 2.

Given the plethora of dominant selectable markers for *Leishmania* gene targeting [13,51,54–56], as well as the capacity to manipulate the purine sources in the cell culture medium, it has been possible to introduce multiple mutations into the purine salvage pathway in many different combinations (Table 2). The ability to generate viable  $\Delta hgprt/\Delta aprt/ak^-$  [9] and  $\Delta xprt/\Delta aprt/ak^-$  [11] *L. donovani*, each with mutations in three of the four salvage enzymes, revealed that the parasite can rely solely on XPRT or HGPRT, respectively, to meet all of its nutritional requirements and genetically validated that exogenous purines are funneled to hypoxanthine and/or xanthine by *L. donovani* prior to incorporation into the parasite nucleotide pools. Strikingly, however, our initial selection strategies did not enable selection of a combined  $\Delta hgprt/\Delta xprt$  genotype [8,9,11]. The  $\Delta hgprt/\Delta xprt$  mutant was, however, eventually isolated in the presence of adenine and dCF, which enabled mutant parasite survival and growth by inhibiting adenine deamination and thereby obliging adenine salvage through APRT. Growth of the  $\Delta hgprt/\Delta xprt$  mutant was conditional upon the presence of dCF and either adenine or adenosine as the purine source, since no other purine supported the survival or growth of the double knockout either in the absence or presence of dCF [11]. Despite the conditional growth phenotype, sub-populations of very slow growing  $\Delta hgprt/\Delta xprt$  cells were selected on plates containing inosine and hypoxanthine [57]. These subpopulations accommodated extrachromosomal amplifications of *APRT* and were, surprisingly, capable of converting hypoxanthine to IMP through APRT, although the  $K_m$  of APRT for hypoxanthine was three orders of magnitude greater than that for adenine [52,57]. The  $\Delta aah$  cell line was also used as a background strain to generate a  $\Delta aah/\Delta hgprt/\Delta xprt$  triple knockout, which unlike the  $\Delta hgprt/\Delta xprt$  mutant, is not dependent on dCF for growth, providing unequivocal evidence that AAH is the cellular target for dCF [10].

## An 'Achilles' Heel' revealed?

The conditionally lethal growth phenotype of the  $\Delta hgprt/\Delta xpirt$  double knockout established that salvageable purines are funneled to substrates of the two missing enzymes. Thus, the majority of purine nucleobases and nucleosides in wild type *L. donovani* are first fluxed to IMP and XMP and then converted to AMP and GMP, respectively (Figures 1 and 2), insinuating that the nucleotide interconversion enzymes, inosine monophosphate dehydrogenase (IMPDH), adenylosuccinate synthetase (ADSS), adenylosuccinate lyase (ASL), GMP reductase (GMPR) and GMP synthase (GMPS) might be essential to the parasite. Using rational selective strategies,  $\Delta impdh$  [58],  $\Delta adss$ ,  $\Delta asl$ , and  $\Delta gmpr$ , null mutants were easily generated under appropriate permissive growth conditions. Permissive and restrictive growth conditions for the  $\Delta adss$ , and  $\Delta asl$  promastigotes were, as anticipated, the same as those for the  $\Delta hgprt/\Delta xpirt$  parasites (J.M. Boitz, *et al.*, unpublished), while xanthine and guanine were permissive and adenine and hypoxanthine restrictive for growth of the  $\Delta impdh$  promastigotes [58], Table 2. Unexpectedly, however, we were unable to create a  $\Delta gmpr$  knockout even with the addition of excess guanine, which we conjectured would circumvent the genetic lesion, in the culture medium. Therefore, we concluded, based on previous genetic and metabolic flux studies [2,10–12], that a homozygous  $\Delta gmpr$  mutation could be lethal to the parasite because of the presence of GDA. GDA would effectively deaminate guanine to xanthine, which in turn would be phosphoribosylated by XPRT to XMP, a dead end substrate for a  $\Delta gmpr$  mutant, thereby starving the mutant parasites for guanylate nucleotides regardless of the nature of the purine supplementation of the growth medium (Figure 2). We proposed, therefore, that introduction of a  $\Delta gda$  mutation into the parasite would drive guanine to GMP through HGPRT to GMP, thereby circumventing the potentially lethal  $\Delta gmpr$  lesion. This hypothesis is currently being tested. The observation that *Leishmania* funnel all salvageable guanine to xanthine, even when XMP accumulates as a dead-end metabolite in  $\Delta gmpr$  cells, points to a lack of regulational control of GDA and XPRT activity. Given that mammalian cells lack XPRT, it is tempting to postulate that the robust activity of the *L. donovani* GDA may have evolved for the benefit of the parasite, since conversion of guanine to xanthine in essence acts to trap this metabolite in a form only usable by the parasite.

## Purine salvage and the purine nutritional environment within the mammalian host

*Leishmania* spp. are digenetic parasites, existing as highly motile, flagellated promastigotes that reside freely within the midgut of the sandfly vector and as amotile amastigotes that replicate within the phagolysosome of the mammalian macrophage. Most of the initial investigations on the purine pathway were undertaken with an attenuated *L. donovani* clone [59] derived from the 1S-2D strain that was originally adapted to axenic culture by Dennis Dwyer [60–62]. Consequently, studies on parasite infectivity and virulence were precluded. Recently, LdBob, a highly virulent, rapidly growing 1S-2D sub-clone that can axenically cycle between promastigotes and amastigotes and that can infect macrophages and mice, has become available [12,63]. LdBob has proven invaluable for directly assessing the nutritional role of purine salvage enzymes in the amastigote and for determining the nature of host purines available for salvage in host cells [61–63].

To determine whether any single purine salvage or interconversion enzyme is essential for purine nutrition and parasite viability in the infectious form of the parasite, the  $\Delta hgprt$ ,  $\Delta aprt$ ,  $\Delta xpirt$ ,  $\Delta aah$ ,  $\Delta impdh$  [10,12,58],  $\Delta adss$ , and  $\Delta asl$  (J. M. Boitz *et al.*, unpublished) mutants, were created within the LdBob background. All of these knockouts retained their ability to transform into axenic amastigotes and exhibited purine growth profiles identical to those of their promastigote counterparts. Therefore, the purine salvage pathway does not

appear to deviate significantly between the two life cycle stages – at least *in vitro*. Likewise the  $\Delta hgprt/\Delta xpirt$  mutant constructed in the LdBob background also exhibited an absolute dependence on dCF and exogenous adenine or adenosine in its axenic amastigote form, strongly implying that AAH is active in both life cycle stages and contrasting to earlier reports indicating that AAH was promastigote-specific [3,10,12].

Several of these mutants have also been evaluated, either singly or in combination, for their impact on the ability of the parasite to infect and replicate within primary murine macrophages or establish and maintain infections in mice. Of the mutants tested thus far, including  $\Delta aah$ ,  $\Delta impdh$  [10,58],  $\Delta adss$ , and  $\Delta asl$  (J. M. Boitz *et al.*, unpublished), only the  $\Delta asl$  mutant exhibits a severely reduced parasite burden in both macrophages and mice. This contrasts with the infectivity phenotype of the  $\Delta adss$  knockout, which also accommodates a genetic lesion in IMP to AMP conversion (Figures 1 and 2) but is only marginally impacted in its capacity to infect macrophages and mice. The discrepancy between the infectivity capacities of the two mutants defective in IMP to AMP synthesis can be explained by the accumulation of adenylosuccinate observed in the  $\Delta asl$  mutant *in vitro*. This implies that the conversion of IMP to adenylosuccinate by ADSS is unregulated and that unchecked adenylosuccinate accumulation is a lethal event. These data also shed light on the host purine environment, signifying that sufficient host-derived adenine or adenosine is available for direct salvage to AMP, which can fulfill the requirement for adenylate nucleotides in the  $\Delta adss$  and  $\Delta asl$  parasites (J. M. Boitz *et al.*, unpublished).

Of the various *L. donovani* strains harboring multiple mutations, only the  $\Delta hgprt/\Delta xpirt$  and  $\Delta aah/\Delta hgprt/\Delta xpirt$  lines have been tested in macrophages and mice. The  $\Delta hgprt/\Delta xpirt$  double knockout exhibited an extremely reduced parasite burden in both primary murine macrophages and mice, but parasite numbers were restored to wild type levels by complementation with *XPRT* expressed in *trans* from an episomal vector [11]. Since *XPRT* is able to metabolize both hypoxanthine and xanthine to the nucleotide level [14], it is unclear whether *XPRT* complementation of the infectivity deficit indicates that the primary purine salvage insufficiency in the  $\Delta hgprt/\Delta xpirt$  null mutant is an inability to convert guanine and xanthine into guanylate nucleotides or a deficit in both guanylate and adenylate nucleotide production. Ongoing investigations to examine the impact of single  $\Delta hgprt$  or  $\Delta xpirt$  mutations on parasite fitness in mice should clarify this point. Although the  $\Delta hgprt/\Delta xpirt$  double knockout was profoundly incapacitated in its ability to infect mice, parasites could still be recovered from livers and spleens after four weeks of infection. Whether persistent parasites were quiescent, dividing slowly, or dying is not clear, since studies from our laboratory indicate that purine-starved promastigotes can persist in a ‘quiescent-like’ state for days and even several weeks in culture [39]. The persistence of the  $\Delta hgprt/\Delta xpirt$  parasites in mice may arise from an increase in *APRT* activity, since *APRT* protein levels were slightly augmented in these cells - consistent with our previous findings in which *APRT* overexpression was shown to suppress the  $\Delta hgprt/\Delta xpirt$  mutation *in vitro*. This is also substantiated by results from macrophage infectivity experiments, where the incapacitating effects of the  $\Delta hgprt/\Delta xpirt$  lesion could be surmounted by the overexpression of *APRT*, restoring infections to near wild type levels. Thus, the infectivity data recapitulates the *in vitro* phenotype for HGPRT and XPRT and suggests that combined lesions in these enzymes severely impact the capacity to infect within the mouse model.

Furthermore, the  $\Delta hgprt/\Delta xpirt$  lesions have also been introduced into the  $\Delta aah$  background, and these triple knockout parasites have been tested for their capacity to infect mice and murine macrophages [10]. Although we expected that the  $\Delta aah$  lesion would preserve host-derived adenine for conversion to AMP by *APRT* (Figures 1 and 2), the  $\Delta aah/\Delta hgprt/\Delta xpirt$  mutant was even more compromised in the murine model than the  $\Delta hgprt/\Delta xpirt$  strain. In fact, no  $\Delta aah/\Delta hgprt/\Delta xpirt$  parasites were recovered from livers or spleens from five

infected mice or from parallel infections in cultured macrophages. Parasite infectivity was fully restored by an episomal copy of *XPRT*, demonstrating that loss of infectivity was not due to the  $\Delta aah$  mutation but rather to the HGPRT and XPRT deficiencies [10].

## Concluding remarks

Metabolic flux and gene replacement studies suggest that, despite a superficial complexity and convolutedness, the majority of purine flux into nucleotides in *Leishmania* is limited to two main routes, HGPRT and XPRT. Thus the salvage of exogenous purine nucleosides and nucleobases is simplified by their robust distillation to HGPRT and XPRT substrates. These studies have also highlighted GMPS as a potential Achilles' heel within the pathway, where due to the robust conversion of guanine to xanthine by GDA, the pharmacological ablation of GMPS activity would effectively pit the metabolism of the parasite against itself to starve them for guanylate nucleotides.

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## Glossary

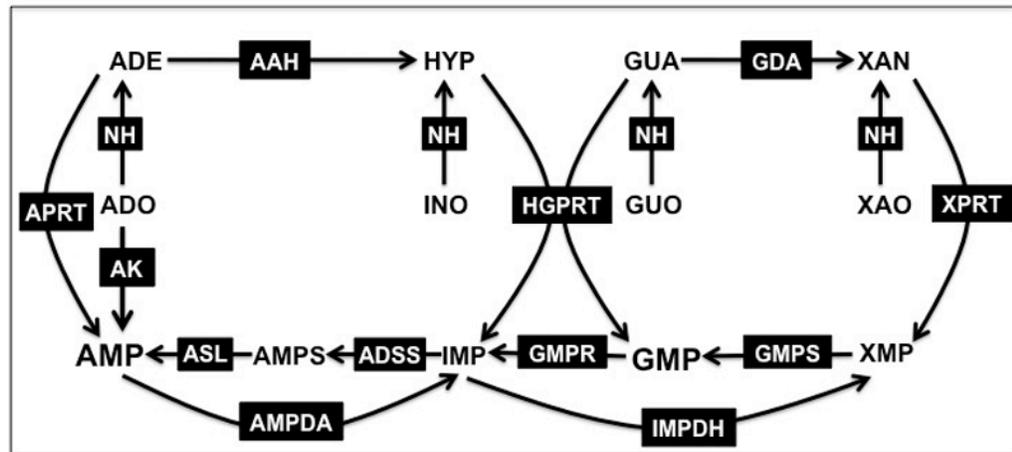
<b>AAH</b>	adenine aminohydrolase
<b>ADSS</b>	adenylosuccinate synthetase
<b>AK</b>	adenosine kinase
<b>AMPDA</b>	AMP deaminase
<b>APRT</b>	adenine phosphoribosyltransferase
<b>ASL</b>	adenylosuccinate lyase
<b>GDA</b>	guanine deaminase
<b>GMPR</b>	GMP reductase
<b>GMPS</b>	GMP synthase
<b>HGPRT</b>	hypoxanthine-guanine phosphoribosyltransferase
<b>IMP</b>	inosine monophosphate
<b>IAGNH</b>	inosine-adenosine-guanosine nucleoside hydrolase
<b>IGNH</b>	inosine-guanosine nucleoside hydrolase
<b>IMPDH</b>	inosine monophosphate dehydrogenase
<b>IUNH</b>	inosine-uridine (or non-specific) nucleoside hydrolase
<b>LdNT</b>	<i>Leishmania donovani</i> Nucleoside or Nucleobase Transporter
<b>PRPP</b>	phosphoribosylpyrophosphate
<b>XMP</b>	xanthine monophosphate
<b>XPRT</b>	xanthine phosphoribosyltransferase

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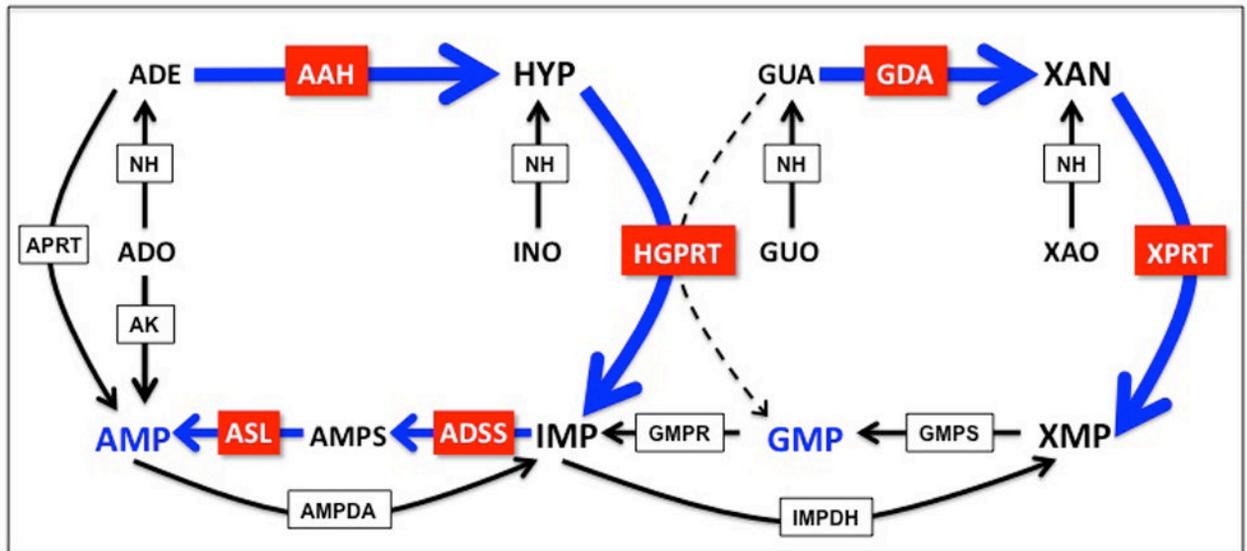
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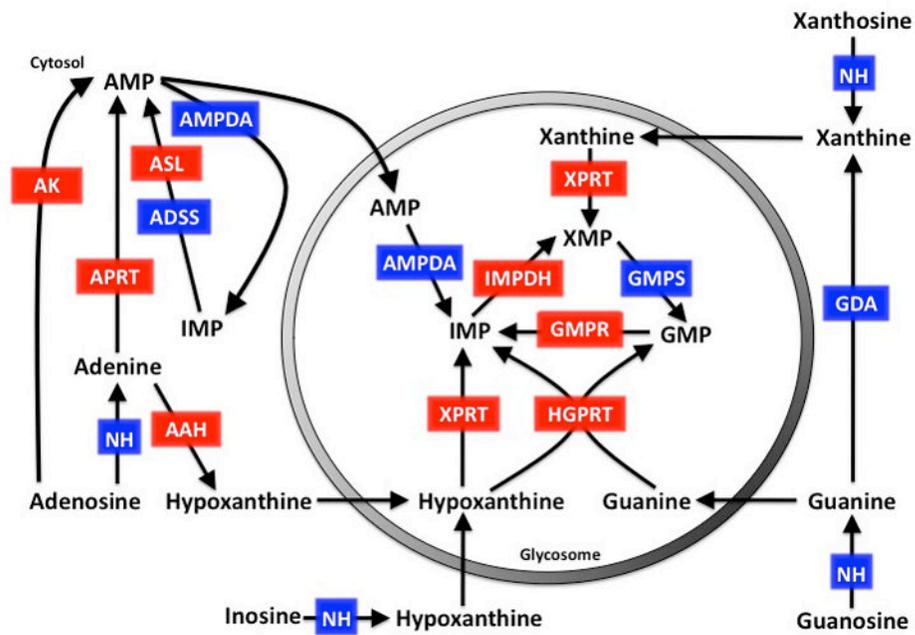
**Figure 1.**

Predicted purine salvage pathway of *Leishmania*. The purine salvage and interconversion enzymes that have been identified in *L. donovani* are depicted. Abbreviations: APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AK, adenosine kinase; AAH, adenine aminohydrolase; GDA, guanine deaminase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; AMPDA, AMP deaminase; IMPDH, inosine monophosphate dehydrogenase; GMPS, GMP synthase; GMPR, GMP reductase; NH, nucleoside hydrolase; ADO, adenosine; ADE, adenine; INO, inosine; HYP, hypoxanthine; GUO, guanosine; GUA, guanine; XAO, xanthosine; XAN, xanthine.



**Figure 2.**

Metabolic flux through the purine salvage pathway in *L. donovani*. Heavy blue arrows highlight the major flux of substrates through the purine salvage pathway as deduced from genetic and biochemical experiments. The minor activities are represented by black arrows, and the dashed line indicates that the phosphoribosylation of guanine most likely does not occur under physiological conditions in *Leishmania*. Abbreviations: APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AK, adenosine kinase; AAH, adenine aminohydrolase; GDA, guanine deaminase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; AMPDA, AMP deaminase; IMPDH, inosine monophosphate dehydrogenase; GMPS, GMP synthase; GMPR, GMP reductase; NH, nucleoside hydrolase; ADO, adenosine; ADE, adenine; INO, inosine; HYP, hypoxanthine; GUO, guanosine; GUA, guanine; XAO, xanthosine; XAN, xanthine.



**Figure I.**

Predicted compartmentalization of the *Leishmania* purine salvage pathway. The gray shaded circle represents the glycosomal membrane and the cytosolic environment is located outside of the circle. Enzymes within the purine salvage pathway that have been experimentally determined to be in the cytosol or the glycosome are shown in red and those that are based on their *in silico* predictions are shown in blue. Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, GMP synthase; GMPR, GMP reductase; APRT, adenine phosphoribosyltransferase; AAH, adenine aminohydrolase; AK, adenosine kinase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; AMPDA, AMP deaminase; GDA, guanine deaminase; NH, nucleoside hydrolase.

Table 1

Accession numbers for all of the known purine salvage and interconversion enzymes of five different *Leishmania* species

Gene	<i>Leishmania</i> species					Refs.
	<i>L. donovani</i>	<i>L. infantum</i>	<i>L. major</i>	<i>L. mexicana</i>	<i>L. braziliensis</i>	
<i>AK</i>	LdBPK_300940.1	LinJ.30.0940	LmjF.30.0880	LmxM.29.0880	LbrM.30.1010	[16,17]
<i>APRT</i>	LdBPK_260120.1	LinJ.26.0120	LmjF.26.0140	LmxM.26.0140	LbrM.26.0130	[16,17]
<i>HGPRT</i>	LdBPK_210980.1	Lind.21.0980	LmjF.21.0845	LmxM.21.0845	LbrM.21.0990	[16,17]
<i>XPRT</i>	LdBPK_210990.1	LinJ.21.0990	LmjF.21.0850	LmxM.21.0850	LbrM.21.1000	[16,17]
<i>AAH</i>	LdBPK_352200.1	LinJ.35.2200	LmjF.35.2160	LmxM.34.2160	LbrM.34.2090	[16,17]
<i>ADSS</i>	LdBPK_131090.1	Lind.13.1090	LmjF.13.1190	LmxM.13.1190	LbrM.13.0970	[16,17]
<i>ASL</i>	LdBPK_040440.1	LinJ.04.0440	LmjF.04.0460	LmxM.04.0460	LbrM.04.0500	[16,17]
<i>GDA</i>	LdBPK_290920.1	LinJ.29.0920	LmjF.29.0867	LmxM.08_29.0867	LbrM.29.0910	[16,17]
<i>GMPS</i>	LdBPK_220013.1	Lind.22.0013	LmjF.22.0110	LmxM.22.0110	LbrM.22.0110	[16,17]
<i>GMPR</i>	LdBPK_170870.1	LinJ.17.0870	LmjF.17.0725	LmxM.17.0725	LbrM.17.0790	[16,17]
<i>IMPDH</i>	LdBPK_191590.1	LinJ.19.1590	LmjF.19.1560	LmxM.19.1560	LbrM.19.1820	[16,17]
<i>IAGNH</i>	LdBPK_292910.1	Lind.29.2910	LmjF.29.2800	LmxM.08_29.2800	LbrM.29.2850	[16,17]
<i>IGNH</i>	LdBPK_140130.1	LinJ.14.0130	LmjF.14.0130	LmxM.14.0130	LbrM.14.0130	[16,17]
<i>IUNH</i>	LdBPK_181570.1	LinJ.18.1570	LmjF.18.1580	LmxM.18.1580	LbrM.18.1610	[16,17]
<i>AMPDA</i>	LdBPK_040270.1	Lind.04.0270	LmjF.04.0280	LmxM.04.0280	LbrM.04.0320	[16,17]
<i>AMPDA</i>	LdBPK_130870.1	LinJ.13.0870	LmjF.13.0980	LmxM.13.0980	LbrM.13.0790	[16,17]
<i>AMPDA</i>	LdBPK_322690.1	LinJ.32.2690	LmjF.32.2550	LmxM.31.2550	LbrM.32.2780	[16,17]
<i>AMPDA</i>	LdBPK_354860.1	Lind.35.4860	LmjF.35.4800	LmxM.34.4800	LbrM.34.4760	[16,17]

Abbreviations: AK, adenosine kinase; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AAH, adenine aminohydrolase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; GDA, guanine deaminase; GMPS, GMP synthase; GMPR, GMP reductase; IMPDH, inosine monophosphate dehydrogenase; IAGNH, inosine, adenosine, guanosine nucleoside hydrolase; IGNH, inosine-guanosine nucleoside hydrolase; IUNH, inosine uridine nucleoside hydrolase; AMPDA, AMP deaminase

Table 2

The capacity of *L. donovani* purine salvage mutants to grow in a range of purine sources

Cell Line	Purine Source											
	ADE	ADE + dCF	ADO	ADO + dCF	GUA	GUO	HYP	INO	XAN	HYP + XAN	HYP + XAN	
Wild Type	+	+	+	+	+	+	+	+	+	+	+	+
<i>Laah</i>	+	+	+	+	+	+	+	+	+	+	+	ND
<i>ak<sup>-</sup></i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>aprt<sup>-</sup></i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>Δaprt</i>	+	-	+	+	+	+	+	+	+	+	+	ND
<i>Δadss</i>	-	+	-	+	-	-	-	-	-	-	-	ND
<i>Δasl</i>	-	+	-	+	-	-	-	-	-	-	-	ND
<i>Δgda</i>	+	+	+	+	+	+	+	+	+	+	+	ND
<i>Δgnpr</i>	+	+	+	+	-	-	+	+	-	+	+	+
<i>Δhgprt</i>	+	+	+	+	+	+	+	+	+	+	+	ND
<i>Δinpdh</i>	-	ND	-	ND	+	+	-	-	+	+	+	+
<i>Δxprt</i>	+	+	+	+	-	+	+	+	+	+	+	ND
<i>aprt<sup>-</sup>/Δxprt</i>	+	ND	+	ND	-	+	+	+	+	+	+	ND
<i>ak<sup>-</sup>/Δaprt</i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>ak<sup>-</sup>/Δhgprt</i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>ak<sup>-</sup>/Δxprt</i>	+	ND	+	ND	-	+	+	+	-	+	+	ND
<i>Δhgprt/Δaprt</i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>Δhgprt/Δxprt</i>	-	+	-	+	-	-	-	-	-	-	-	ND
<i>Δaah/Δadss</i>	+	+	-	+	-	-	-	-	-	-	-	ND
<i>Δgnpr/Δinpdh</i>	-	-	-	-	-	-	-	-	-	-	-	+
<i>Δhgprt/Δaprt/ak<sup>-</sup></i>	+	ND	+	ND	+	+	+	+	+	+	+	ND
<i>Δxprt/Δaprt/ak<sup>-</sup></i>	+	ND	+	ND	-	+	+	+	+	+	+	ND
<i>Laah/Δhgprt/Δxprt</i>	+	+	+	+	-	-	-	-	-	-	-	ND

Abbreviations: (+), continuous growth could be maintained; (-), growth could not be sustained; (ND), growth not determined; ADE, adenine; dCF, 2' deoxycoformycin; ADO, adenosine; GUA, guanine; GUO, guanosine; HYP, hypoxanthine; INO, inosine; XAN, xanthosine