# CHAPTER 10

# Antiparasitic Chemotherapy:
## Tinkering with the Purine Salvage Pathway

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## Summary

Distinguishable differences between infecting organisms and their respective hosts with respect to metabolism and macromolecular structure provide scopes for detailed characterization of target proteins and/or macromolecules as the focus for the development of selective inhibitors. In order to develop a rational approach to antiparasitic chemotherapy, finding differences in the biochemical pathways of the parasite with respect to the host it infects is therefore of primary importance. Like most parasitic protozoan, the genus *Leishmania* is an obligate auxotroph of purines and hence for requirement of purine bases depends on its own purine salvage pathways.

Among various purine acquisition routes used by the parasite, the pathway involved in assimilation of adenosine nucleotide is unique and differs significantly in the extracellular form of the parasite (promastigotes) from its corresponding intracellular form (amastigotes). Adenosine kinase (AdK) is the gateway enzyme of this pathway and displays stage-specific activity pattern. Therefore, understanding the catalytic mechanism of the enzyme, its structural complexities and mode of its regulation have emerged as one of the major areas of investigation. This review, in general, discusses possible strategies to validate several purine salvage enzymes as targets for chemotherapeutic manipulation with special reference to adenosine kinase of *Leishmania donovani.*

Systemic endotheliosis, commonly known as Kala-azar in India, is caused by the parasitic protozoon *Leishmania donovani.* The spread of leishmaniases follows the distribution of these vectors in the temperate, tropical and subtropical regions of the world leading to loss of thousands of human lives.[1] WHO has declared leishmaniasis among one of the six major diseases namely leishmaniasis, malaria, amoebiasis, filariasis, Chagas disease and schistosomiasis in its Special Programme for Research and Training in Tropical Diseases. Strategies for better prophylaxis and urgent therapies must be therefore devised to control this menace among poor and under privileged population. However, the possible availability of antiparasitic vaccines appears remote in near future. Therefore, chemotherapy remains the mainstay for the treatment of most parasitic diseases.

Selectivity of an antiparasitic compound must depend upon its mode of specific inhibition of parasite replication leaving host processes unaffected. In principle, these agents are expected to exert their selective actions against growth of the invading organisms by having one or both of the following properties:

i. Selective activation of compounds in question by enzyme (s) from the invading organisms, which are not present in the uninfected cells.
ii. Selective inhibition of vital enzyme(s), which are essential for replication of the parasites.

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In order to design specific compounds with the above characteristics, it is essential to have a thorough knowledge of the properties of the enzyme(s) and/or macromolecules which are unique to the parasite. Phylogenetic studies suggested that trypanosomatid parasites are relatively early-branching eukaryotic cells and indeed their cellular organization differs considerably from their mammalian hosts counterpart.[2] Various enzymes, metabolites or proteins identified in parasites and known to be absent from or strikingly different in the mammalian hosts were considered as ideal drug targets. Among the various metabolic pathways that are presently being studied for their prospects to be exploited as the target for chemotherapeutic manipulation, the most important are (i) purine salvage (ii) polyamine and thiol metabolism (iii) folate biosynthesis (iv) DNA replication (v) glycolytic and (vi) fatty acid biosynthetic pathways etc. A number of excellent reviews, describing the prospects and efficacies of these pathways, already exist in the literature.[3-5] Our laboratory is engaged in studying the pathways responsible for synthesis and assimilation of purine nucleotides in the parasitic protozoon *Leishmania donovani.* Therefore, we shall, for the constraint of space, try to restrict the discussion mostly with the purine salvage pathways of various *Leishmania* parasites with particular reference to the unique features of one of the enzymes of the purine salvage pathway viz AdK and its prospects as the chemotherapeutic target. However, contributions of other workers will also be discussed whenever essential and analogy will be drawn in order to make the reading coherent.

The *Leishmania* genus goes through a dimorphic life cycle.[3,4] It exists as a promastigote (extracellular form) in the sand fly vector but is converted to an amastigote (intracellular form) upon entry into mammalian macrophages. During this transformation process, the activities of a large number of proteins and/or enzymes have been reported to be stage-specifically altered and hence they could be prospective targets for development of chemotherapeutic regimen based on the exploitable differences of the parasitic proteins from their respective host counterpart.[6-12]

## General Strategies for Development and Characterisation of Drug Targets in Trypanosomatids

The traditional approach to the development of new antiparasitic compounds consists of screening of a large number of compounds or extracts containing natural bio-active products against particular pathogens. This random approach is conducted without prior knowledge of either the molecular target(s) within the pathogen or the mode of action of the drugs. A large majority of antiparasitic compounds in use today were developed using these strategies. Subsequently, the molecular targets of few of these drugs viz. chloroguanide, pyrimethamine and trimethoprim, have been identified.[13]

However, the more contemporary and rational approach to drug discovery began with the identification of molecular targets within the parasite. Targets are selected based on their essential functions in the survival of the cell. In the recent years gene knockout approach is the routine procedure employed for studying a particular gene-function and has thus become a method of choice for target validation. Following selection and validation of the target protein, the objectives are to identify the details of their molecular structures and/or functions that can be exploited to design compounds inhibitory to the target molecule in question. The structure of the target can be studied by crystallisation of the protein or may be modelled using three-dimensional coordinates of amino acids from related or homologous protein whose crystal structure is known.[14] This structure-based approach to the design of compounds has benefits beyond the discovery of selective potent inhibitors as it provides an additional advantage for the prediction and resolution of drug-resistance problems. The process does not have to wait for the appearance of drug-resistant strains. Instead, possible drug-resistant strains can be generated in vitro using low concentrations of drugs. The development of resistance can then be studied by identifying possible mutations in the target proteins.[13,15] Subsequently, the mutations can be analysed using interactive computer graphic display systems, which visualises changes in the three-dimensional structure of the protein. These studies, apart from revealing the possible

mechanism for altered drug binding, also may suggest ways for designing modified compounds for development of second generation of drugs for treatment of drug-resistant strains.

Because purine salvage pathway has been widely accepted as one of the plausible targets for the development chemotherapeutic regimen in most parasites including *L. donovani*, the subsequent sections will highlight the known complexities of this pathway and point out the differences observed during transformation of the parasite from its extracellular to its intracellular form.

## Acquisition and Assimilation of Purines in *L. donovani* Promastigotes

It is now well established that all parasitic protozoa, including *L. donovani*, as opposed to most of the mammalian cells, lack the ability to synthesize purines de novo and thus use their own unique complement of salvage enzyme system to scavenge purines from the host.[16-19] Only nucleosides viz. adenosine, guanosine and inosine and their analogs viz. tubercidin, iodotubercidine etc. or nucleobases (adenine, xanthine and guanine) can be taken up by the cell surface transporters. The presence of two such specific cell surface nucleobase/nucleoside transporters (viz. LdNT1 and LdNT2) has been well documented for leishmania species.[20-22] They mediate the uptake of purine nucleosides as well as some purine analogs but with different specificities. However, host nucleotides must have to be converted into respective nucleosides prior to uptake. This task is accomplished by unique cell surface 3'-nucleotidase/nucleases.[23-26] The flow sheet diagram depicts the known pathways by which purine bases are scavenged and/or assimilated in *Leishmania* (Fig. 1). The key enzymes involved in this process

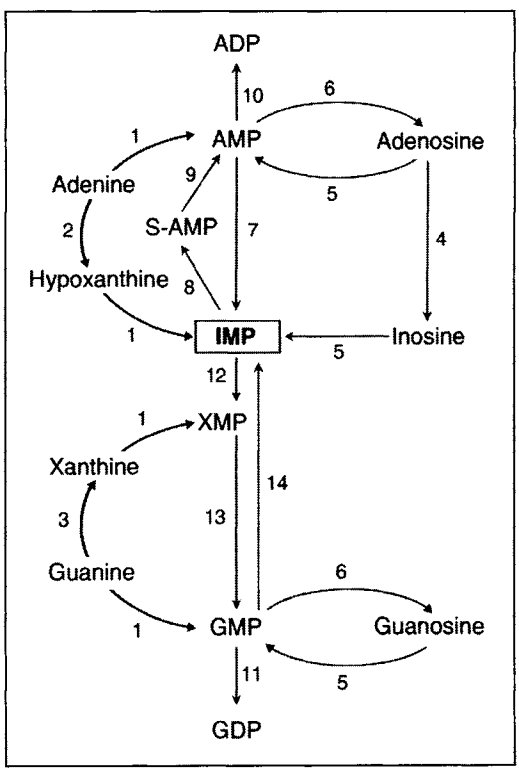


Figure 1. Purine salvage pathways of *Leishmania species*. Enzymes: 1) phosphoribosyltransferase; 2) adenine deaminase; 3) guanine deaminase; 4) adenosine deaminase; 5) nucleoside kinase; 6, nucleotidase; 7) AMP deaminase; 8) adenylosuccinate synthetase; 9) adenylosuccinate lyase; 10) AMP kinase; 11) GMP kinase; 12) IMP dehydrogenase; 13) GMP synthetase; 14) GMP reductase.

are adenine deaminase and guanine deaminase, which convert adenine and guanine to hypoxanthine and xanthine respectively. Interestingly, phosphorylase activity has not yet been detected in the *Leishmania.* Therefore, phosphoribosyl transferase (PRTase) activity appears to play a central role in the salvage of these purine bases.[27] Till date, three such PRTase activities viz. adenine phosphoribosyl transferase (APRTase), hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) and xanthine phosphoribosyl transferase (XPRTase) have been identified and characterised in *Leishmania.*[27] These PRTases of the parasite then convert hypoxanthine and xanthine to inosine monophosphate (IMP) and xanthine monophosphate respectively. The enzyme XPRTase of *L. donovani* has been the focus of attention for a long time as it is absent in mammalian systems.[28] Moreover, since analogs of naturally occurring purine bases, known as subversive substrates, can function as prodrugs and enter nucleotide pool with lethal effect, this group of enzymes have stimulated considerable therapeutic interest with regard to a spectrum of parasitic diseases.

A stage-specific difference in the activities of enzymes is another characteristic of some *Leishmania* species, with promastigotes containing adenine deaminase and amastigotes containing adenosine deaminase. IMP formed in the cell can be converted to AMP by adenylosuccinate synthetase and adenylosuccinate lyase whereas XMP is converted to IMP by GMP synthetase and GMP reductase. Moreover, IMP dehydrogenase has also the ability to convert XMP to GMP.[27]

Nucleosides, following entry into the cell, are converted to mononucleotides by either phosphotransferases or by nucleoside kinases. Phosphotransferase activity has been detected in the extracts of *L. donovani* but these enzymes have been found to utilize only inosine analogs as the substrate.[29] Adenosine kinase (AdK), that directly phosphorylates adenosine (Ado) to AMP, is present both in promastigote and in amastigote of *L. donovani* and *L. mexicana mexicana* whereas guanosine kinase is present only in *L. m. mexicana.* Two other kinases viz. inosine and xanthosine kinases have also been detected in very low amounts. Adenosine deaminase, an important enzyme in mammalian cells, is however present in *Leishmania* amastigotes only.

## Purine Metabolism in *L. donovani* Amastigotes

Purine metabolism in *L. donovani* and *L. m. mexicana* amastigotes has been extensively studied by Looker et al[8] and Hassan and Coombs.[30] The pathways of utilization of guanine, xanthine, hypoxanthine and their respective nucleosides are similar in both forms of the parasites. However, adenosine metabolism in *L. donovani* amastigotes differs markedly from that in promastigotes. In this connection, it may be mentioned that although a stage-specifically expressed adenosine transporter has been reported in amastigotes, confirmation of its existence is still awaiting.[31] In any case, following uptake in the amastigote, adenosine is deaminated to inosine by adenosine deaminase, not detectable in promastigotes. Subsequently, inosine is cleaved to hypoxanthine. However, adenine deaminase, that is known to deaminate adenine to hypoxanthine in the promastigote, is not present in the amastigote. In contrast, the activity of AdK in the amastigote shows 50-fold increase over the activity observed in the promastigote.[8]

It therefore appears that the *Leishmania* parasites possess multiple routes for salvaging purines and all the purine bases are interconvertible with an apparent branch point at IMP. Hence, the *Leishmania* species, unlike some other protozoa, when cultured in vitro, do not require any particular purine base for growth.

## Purine Salvage Enzymes as Targets for Structure-Based Inhibitor Design

The potential of the purine phosphoribosyl transferases (APRTase, XPRTase, HGPRTase) and AdK as targets for antiparasitic chemotherapy stems from the major role of these enzymes in purine acquisition by the trypanosomatid parasites. However, because of the existence of various alternative purine salvage pathways (Fig. 1), it is conceivable that inhibition of a single enzyme would not kill the parasite. This has been confirmed by elaborate genetic investigation

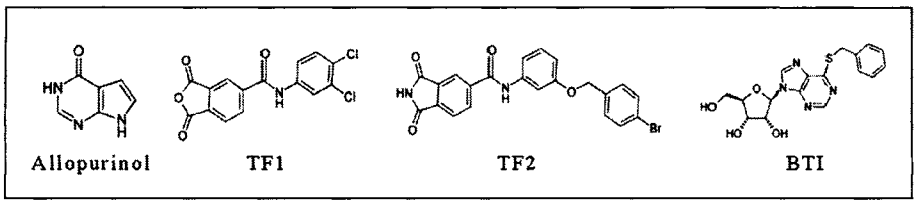


Figure 2. Chemical structures of various inhibitors of HGPRTase and AdK.

of *L. donovani* and *T. gondii*, suggesting that neither of these enzymes are essential for the parasite viability. In this connection it is to be noted that mutant *L. donovani* promastigotes lacking HGPRTase, APRTase and AdK, either singly or in any combination, can retain the capacity to proliferate in completely defined medium in which the sole exogenous purine source is any of the four naturally occurring nucleobases, hypoxanthine, xanthine, guanine or adenine or the nucleosides adenosine, inosine or guanosine, provided it has active XPRTase.[32] How knocking out of these genes will affect the survival of intracellular amastigotes is however remained to be seen. Therefore, it appears that a single chemotherapeutic agent or a combination chemotherapy targeting more than one enzyme would be ideal for successfully blocking the purine acquisition of the parasites.

Till date most of the structure-based inhibitor design strategies that target the purine salvage pathway of the parasite have been directed towards the HGPRTase. Owing to differences in the substrate specificity, the HGPRTase from *Leishmania* and trypanosomes,[33-35] in contrast to their host counterpart, phosphoribosylates antiparasitic pyrazolopyrimidines like allopurinol (Fig. 2), which subsequently is incorporated into the nucleic acids causing selective death of the parasite.[34,35] Allopurinol alone or in combination with other drugs, has been proved to be effective against cutaneous[36] and visceral leishmaniasis.[37] Solution of a number of apo, ion and product bound crystal structures of HGPRTase[38-40] reveals a closed active site that provides well-defined target for computational drug discovery. The structure-based docking method provided a remarkably efficient means for the identification of inhibitors targeting trypanosomal HGPRTase.[41] The inhibition constants of the lead inhibitors were very low with trypanostatic activity in cell culture.[41] Based on X-ray structure of *Tritrichomonas foetus* HGPRTase,[38] isatin and phthalic anhydride were also identified as two novel scaffolds capable of mimicking the substrate purine base and acting as competitive inhibitors of the target enzyme without any detectable effect on the human HGPRTase. TF1 (phthalic anhydride derivative) and TF2 (phthalimide derivative) (Fig. 2) were shown to be effective in killing the cultured parasites and the parasite growth inhibition could be reversed by addition of the natural substrate hypoxanthine to the culture medium.[42,43] These findings demonstrated the success of a structure-based computational approach whereby a molecule identified by computer-based means can be rationally modified to produce potent inhibitors of a chosen target enzyme and may provide useful starting point for drug design for the treatment of different parasitic diseases.

## Prospects of Adenosine Kinase (AdK) as the Drug Target

Among the plethora of purine salvage enzymes in the trypanosomatid parasites, AdK is also being assessed as one of the potential chemotherapeutic targets for treating various parasitic diseases especially leishmaniasis[5] and toxoplasmosis.[5,44] Cohen et al[45] demonstrated that adenylate nucleotide pool is the major source of host purines in all mammalian cells. Since nucleotides do not enter cells readily, they are cleaved by different nucleotidases located on the surface of the parasites and adenine nucleoside is probably the first nucleoside to permeate the plasma membrane of the parasite,[23-25] which is then converted to its nucleotides and other nucleotides. Being the most active purine salvage enzyme, AdK reaction is the main route of adenosine metabolism in *T. gondii*.[46-48] This results in preferential incorporation of adenosine into adenine nucleotides by at least a 10-fold higher rate than that of

any other purine precursor tested.[46,49] Mutant *L. donovani*, lacking AdK, incorporated 25% of the adenosine, indicating 75% of incorporated adenosine is directly phosphorylated by AdK in *L. donovani* promastigotes.[50] Another report indicated that, during transformation of promastigote to amastigote, AdK was stimulated almost 50-fold[8] and thus was suggested to play key role in the process.[51] A very recent study showed that AdK and HXGPRTase provide the only two physiologically relevant routes for purine acquisition in *T. gondii*. However, AdK knock-out parasites exhibited a greater fitness defect than HXGPRTase mutants, arguing that flux through AdK is greater than HXGPRTase.[52]

Structure-activity relationships as well as biochemical and metabolic studies established that the substrate specificity of *T. gondii* AdK differs significantly from those of the human enzyme. It was also demonstrated that AdK from *T. gondii*, as opposed to its host counterpart, preferentially metabolizes 6-benzylthioinosine (BTI) (Fig. 2) to the nucleotide level, which eventually acts as the toxic subversive substrate for the parasite.[5,44,53] Subsequently, several new classes of BTI analogues were synthesized by structure-based lead optimization, leading to further improvement of its antitoxoplasmic efficacy.[54,55] These findings are consistent with the notion that AdK indeed is a key purine salvage enzyme of the *Leishmania* and *Toxoplasma* species. Hence, understanding the reaction mechanism of AdK at the molecular level could be important both from a fundamental point of view as well as in the hope that detailed knowledge of the enzyme may provide relevant information necessary for designing specific inhibitors.

However, the parasitic AdK is one of the most under-exploited purine salvage enzymes as far as its structure-based inhibitor design is concerned. Lack of enough structural information about its active site and insufficient knowledge of the amino acid residues involved in the reaction mechanism have led to such an impasse. Recently however, the X-ray crystal structures of AdK from human and *T. gondii* have been solved.[56,57] Our laboratory has been working on the biochemistry of *L. donovani* AdK (LdAdK) for over two decades and has been a major contributor in unearthing various information regarding its reaction kinetics.[51,58,59] The enzyme from *L. donovani* has also been cloned and expressed, thereby providing workers in this field the necessary impetus to undertake structure-function analysis of the enzyme in a systematic manner.[60]

## General Biochemical Properties of the *L. donovani* AdK

The enzyme from *L. donovani* is a 345-residue monomer of 38 KDa with pI of 8.8, sharply different from the pI (4.5-5.9) determined for AdK from other higher eukaryotic sources and is immunologically distinct from AdK of other sources.[51,58] The enzyme has a pH optimum of 7.5 and the activity is dependent on the optimum ATP-$Mg2^{+}$ ratio. Studies showed that whereas the higher eukaryotic AdKs are prone to inhibition at high Ado and $Mg^{2+}$ concentrations, LdAdK is refractory to such inhibition. In contrast, LdAdK is much more sensitive to inhibition by ATP.[51,59] However, despite these biochemical differences, the parasite enzyme, similar to other AdKs, is regulated by both its products, AMP and ADP.[59,61] LdAdK follows the typical sequential bi-substrate kinetics in which binding of Ado to the enzyme occurs prior to ATP binding with the release of AMP at the end.[59]

## Structure of AdK from Different Sources

AdK sequences from mammalian sources show more than 90% amino acid identity among themselves.[62] Interestingly however, the translated amino acid sequences of enzymes from different sources bear no sequence similarity with other well-characterized nucleoside and nucleotide kinases, thus setting it apart from the family of other structurally and functionally related proteins. However, of particular interest is the two regions of AdK that has been found to be homologous with the members of the PfkB (phosphofructose kinase B) family of carbohydrate kinases viz ribokinase, inosine-guanosine kinase, fructokinase and 1-phosphofructokinase. Members of this family are characterized by the presence of two common sequence motifs that includes a highly conserved di-glycine motif located near the N-terminal end and a DTXGAGD motif, positioned near the C-terminus.[63,64]

Recently, the structure of AdK from human and *T. gondii* has been solved.[56,57] These high resolution structures were determined for the apo enzyme, AdK: Ado complex,[56,57] as well as the Ado: AMP-PCP (a nonhydrolysable ATP analogue) bound enzyme.[57] These findings revealed that the enzyme consists of two unequal-sized domains. The large domain is a three-layered sandwich of α helices and β sheets over which the small domain forms a lid.[65] The cleft formed between the two domains constitutes the catalytic site where several amino acids, probably responsible for Ado and ATP binding, are located. Although the ATP binding site of the enzyme from two sources is quite different, their Ado binding pockets are structurally similar. The location of the magnesium ion between the α and β phosphate of ATP is unusual and differs from several other kinases in which the cation is located between the β and γ phosphates. Nevertheless, the overall structure is similar to the reported structure of *E. coli* ribokinase, the first reported structure in the family of carbohydrate kinases.[66] Structures of human AdK and *E. coli* RK superimposed nicely with an RMS deviation of 2.4 Å, even though the sequence identity between them is only 22%.[56] In the overlapped structures, the ribose ligand of RK superimposed on the ribosyl group of Ado 1 and the adenosine portion of the ADP ligand in RK superimposes on Ado 2. This comparison provided strong evidence that Ado 1 exists in the binding site used for the nucleoside undergoing phosphorylation and that Ado 2 occupies the ATP/ADP-binding site. This was further unambiguously proved by analyzing the AMP-PCP bound structure of *T. gondii* AdK.[57]

Comparison of the structures of *T. gondii* AdK, in presence and in absence of the substrate further revealed a novel catalytic mechanism that involved both global and local changes in the protein structure upon binding of Ado. The most striking among them is the Ado-induced 30° hinge bending motion leading to domain closure. It was predicted that a GG conformational switch was responsible for this gross structural change that placed the enzyme in its precatalytic conformation.[57] Apart from these changes, other additional local structural changes were also shown to be induced by ATP binding. As a result of these transitions, an anion hole is created. In general, the most extensively characterized kinase anion hole is the mononucleotide binding motif or P-loop, which contains the consensus sequence GXX(G/X)XGK(S/T).[67] This motif has been observed in a number of enzymes that include adenylate kinase,[68,69] RecA,[70] elongation factor Tu[71] and p21ras[72] and also in the protein kinases.[73] But the AdK P-loop heptad, DTXGAGD, that encompasses the second ribokinase fingerprint region, is completely different and thus defined a new kinase anion hole motif.[57] Therefore, it is most likely that the synergistic substrate-induced structural changes lead to optimal juxtaposition of the substrates for the catalytic reaction between the adenosine 5'-hydroxyl oxygen atom and the ATP γ-phosphate. Both the structures indicated that the 5'-hydroxyl of adenosine is near to and in reasonable alignment with the γ-phosphate of ATP suggesting an in-line $S_N2$ displacement reaction.[56,57] Despite these structure-based predictions, the actual role of the active site residues involved either in the process of phosphate transfer or substrate binding remains to be biochemically validated.

## Sequence Characteristics of LdAdK Gene and Homology Model-Based Structural Analysis of the Protein

Sequence comparison of the 5'-noncoding region of the AdK gene of *L. donovani* with its corresponding mRNA confirmed that, like other kinetoplastida genes, LdAdK transcript is processed post-transcriptionally following addition of the mini-exon at the 5' end of the mRNA. Furthermore, no consensus eukaryotic promoter sequences such as TATA or CCAAT could be identified upstream of the initiation codon,[74] a finding consistent with other kinetoplastida genes.

Homology alignment studies revealed that LdAdK has only about 40 and 31% identity with human and *T. gondii* enzymes respectively (Fig. 3A). However, despite this limited identity, LdAdK possesses all the characteristics typical of AdK from all known sources. First, similar to that of other AdKs, LdAdK lacks the consensus P-loop motif[60] and secondly, LdAdK harbors two amino acid sequence motifs that are distinctive of the PfkB family of carbohydrate

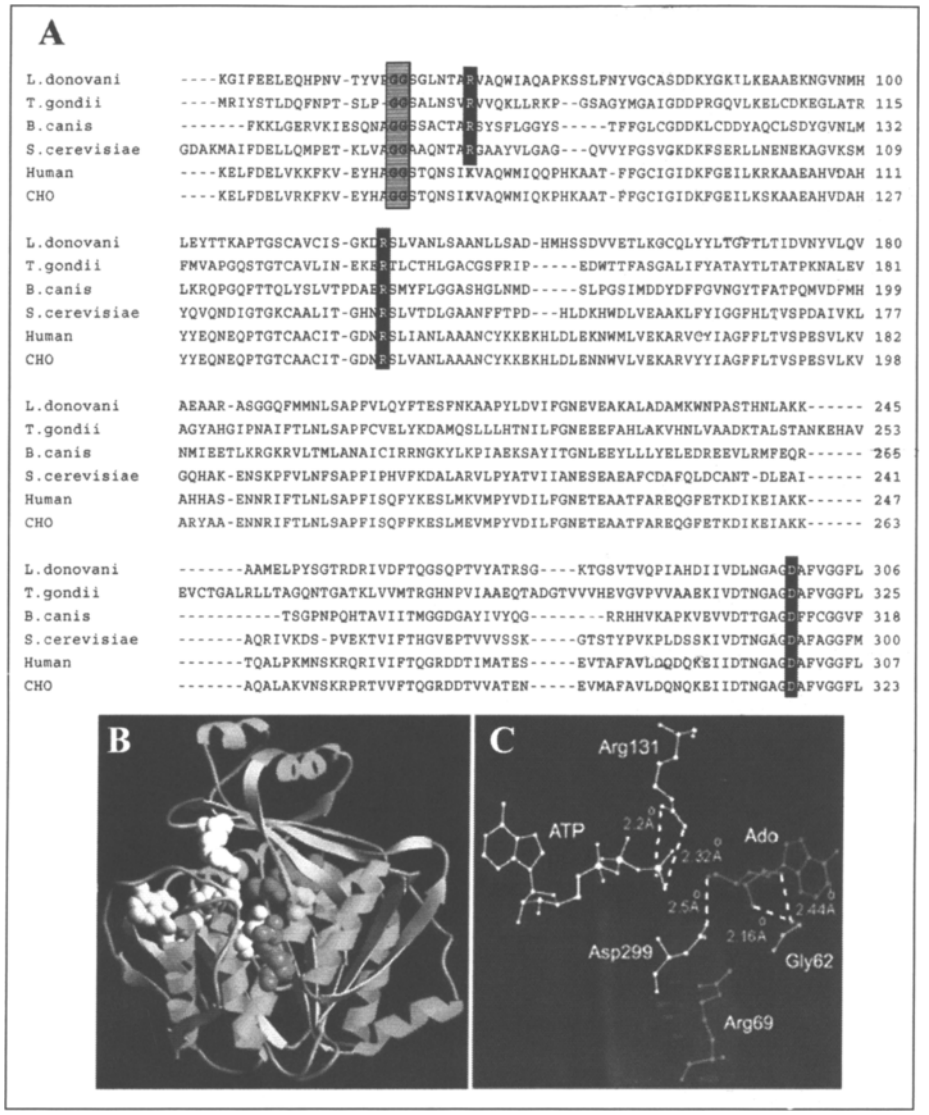


Figure 3. Amino acid sequence alignment and homology modeling of LdAdK. A) alignment of LdAdK amino acid sequence with that of *T. gondii* (AAF01261), *B. canis* (CAA11263), *S. cerevisiae* (P47143) human (AAA97893) and Chinese hamster ovary (AAA91649) AdK (numbers in the parenthesis indicate NCBI database accession numbers for respective proteins). Boxes indicate amino acid signature motifs distinctive of this family of proteins. The residues selected for site-directed mutagenesis i.e., Gly-62, Arg-69 and Arg-131 and Asp-299 of LdAdK are indicated in bold. B) homology model showing the overall structure of LdAdK with α-helices drawn as ribbons and β-strands as arrows. Spatial position of Adenosine (magenta), ATP (yellow), Gly-62 (cyan), Arg-69 (red), Arg-131 (white) and Asp-299 (pink) are shown in space-fill model. C) Zoomed picture of the active site residues with inter-atomic distances shown by dotted lines. Reproduced with permission from: Datta R et al, Biochem J 2005; 387:591-600; ©2005 The Biochemical Society.[78] A color version of this figure is available online at www.Eurekah.com.

kinases.[63,64] Studies further suggested that similar to other carbohydrate kinases, the $Gly_{61}$-$Gly_{62}$ structural motif of LdAdK is probably essential for maintenance of its conformational flexibility. Of the seven arginine residues present in LdAdK, only $Arg_{131}$, corresponding to $Arg_{136}$ and $Arg_{132}$ of *T. gondi* and human AdK respectively, is absolutely conserved. Interestingly, another arginine residue, located at the 69th position of LdAdK seems to be conserved mostly among lower eukaryotes whereas in enzymes from higher eukaryotes this residue is replaced with lysine.[60,62,75-77] Among the acidic residues, $Asp_{299}$ of LdAdK, corresponding to $Asp_{318}$ and $Asp_{300}$ of *T. gondi* and human AdK and located on the second fingerprint motif (DTXGAGD) of ribokinase family, was retained. Likewise, $Asp_{16}$ of LdAdK was also strictly conserved with corresponding amino acid residues $Asp_{24}$ and $Asp_{18}$ of *T. gondii* and human AdK respectively.[65]

In the absence of experimentally determined structure of LdAdK, the three dimensional model, constructed on the basis of the sequence alignment and available coordinates from the human and *T.gondii* AdK crystal structures, showed a high level of overall structural and active-site geometrical symmetry among themselves (Fig. 3B). The ribbon diagram of the model shows that like AdK from other sources, LdAdK appears to be organized into two domains: one of the domains (large domain), consisting of a three-layered sandwich of ten α-helices and nine β-strands, is connected by four peptide segments to the smaller lid domain, consisting of five β-strands running perpendicular to two α-helices.

## Identification of Potential Amino Acid Residues Involved in Catalysis

Taking advantage of the spatial coordinates of the model, the search for the amino acid residues within the interacting distance (≥3.5 Å) of the substrates, was made. In Table 1, residues of LdAdK that are within the interacting distance of either the base, Ado, or the ribose moiety of Ado and ATP or the phosphate group of ATP are listed.

From the analysis, the terminal phosphate group of ATP and the 5'-OH group of the ribose moiety of Ado appeared to be 1.68 Å, close enough for a direct in-line phosphate transfer. The structure further shows that $Gly_{62}$ is located underneath the Ado-binding site of the peptide connecting β-4 sheet of the small domain with the α-3 helix of the large domain (Fig. 3C). Moreover, the peptide N of $Gly_{62}$ is 2.44 Å and 2.16 Å away from the O2' and O3' group of the adenosyl ribose respectively, suggesting its possible role in Ado binding. Of the seven arginine residues present in the protein, only $Arg_{131}$, located on the β-8 sheet of the small domain, appeared to be spatially close to the active site. In fact, its NH1 and NH2 groups were found to be at potentially H-bonding distance of 2.2 Å and 2.3 Å respectively from the O2G and O3G groups of the terminal phosphate of ATP, an observation distinctly different from that of *T. gondii* AdK where, instead of two terminal amino

***Table 1. Hydrogen bonds and close contacts (≤3.5 Å) between the purine or ribose subsite of adenosine and amino acid residues of LdAdK in the modeled structure***

| Purine | | Å | Ribose | | Å |
|---|---|---|---|---|---|
| N1 | Asn12 ND2 | 2.81 | O2' | Asp16 OD1 | 2.67 |
| N1 | Phe168 CB | 3.11 | O2' | Gly62 N | 3.24 |
| N1 | Phe168 CG | 3.28 | O3' | Gly62 N | 2.96 |
| C2 | Phe168 CD1 | 3.45 | O3' | Gly62 CA | 3.48 |
| N3 | Ser63 N | 2.86 | O3' | Gly62 C | 3.25 |
| C6 | Phe168 CG | 3.33 | O3' | Asn66 ND2 | 2.94 |
| N6 | Phe168 CD2 | 3.44 | C5' | Asn295 C | 3.31 |
| N7 | Ala135 CB | 3.40 | C5' | Asn295 O | 3.22 |
| | | | O5' | Asp299 OD2 | 2.50 |

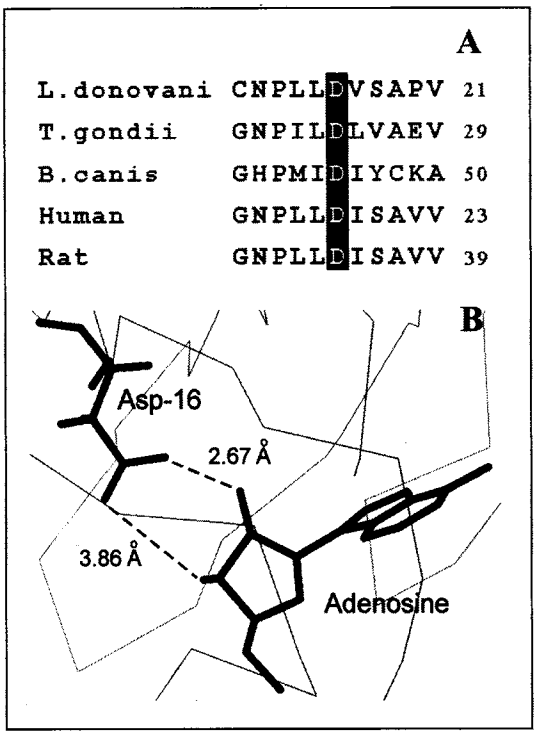


Figure 4. Sequence alignment and close-up view of the Ado-binding site of LdAdK. A) alignment of the N-terminal region of LdAdK amino acid sequence with that from *T.gondii* (AAF01261), *Babesia canis* (CAA11263), human (AAA97893), and rat (AAH81712) showed the invariant aspartate residue (numbers in parentheses indicate GenBank® accession numbers for respective proteins). B) structural model illustrating the position of Asp-16 relative to the bound Ado, dashed line depicts the possible interaction between the Asp-16 carboxyl group and ribose hydroxyls of Ado. Reproduced with permission from: Datta R et al, Biochem J 2006; 394:35-42; ©2006 The Biochemical Society.[63]

groups, NH1 and NH2 were postulated to be interacting with the terminal phosphate.[57] However, the spatial location of the Arg $_{69}$, located on the α-3 helix of the large domain, was found to be quite far away from either of the substrates (>10.0 Å). Apart from these residues, the carboxy side chain of $Asp_{16}$ and $Asp_{299}$ are also proximally located to the ribosyl O2', O3' and O5' hydroxy group of Ado respectively (Figs. 3 and 4). Availability of these structural details allowed initiation of biochemical studies with regard to mechanisms of substrate binding, phosphate transfer and enzyme regulation.

## Mechanism of Ado Binding

Crystal structures of human and *T. gondii* AdK demonstrated that the side chains of $Asp_{18}$ and $Asp_{24}$ respectively (the sequence homologous to $Asp_{16}$ of LdAdK) formed hydrogen bonds with both O2' and O3' ribose hydroxyls.[57] In the modelled LdAdK, its $Asp_{16}$ also points towards the adenosyl ribose (Fig. 4) and is proximal to its O2' and O3' groups. Moreover, comparison of the Ado-binding site of LdAdK and ribose binding site of ribokinase revealed that an Asp residue is conserved in both AdK and RK.[66] Structure-guided mutational analysis of the $Asp_{16}$ mutant demonstrated total obliteration of Ado binding to the enzyme, thereby indicating indispensability of the $Asp_{16}$ residue in Ado binding. Furthermore, possibility of the formation of a bidentate hydrogen bond between $Asp_{16}$ and the adenosyl ribose has also been proposed.[65]

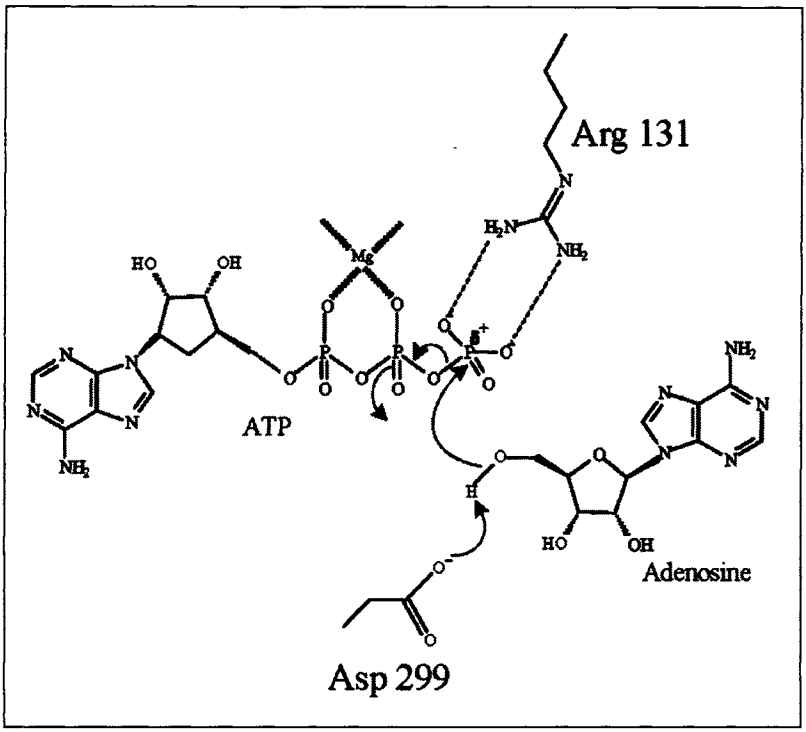


Figure 5. Schematic representation of the possible reaction mechanism of LdAdK; Ado-induced domain rotation around the flexible diglycine motif (Gly-61-Gly-62) places the enzyme in precatalytic conformation. ATP binding causes further conformational changes, resulting in the initiation of a series of events in which Asp-299 first withdraws a proton from the 5' hydroxy group of Ado (solid line) followed by a direct nucleophilic attack on the γ-phosphate of ATP (broken line). The resulting quinquivalent transition state is stabilized by Arg-131. Arg-131 also increases the electrophilicity (δ+) of the γ-phosphorus group. Reproduced with permission from: Datta R et al, Biochem J 2005; 387:591-600; ©2005 The Biochemical Society.[78]

## Mechanism of Phosphate Transfer

Structural information and mutational analysis coupled with chemical modification of some of these residues led to development of a concerted mechanism for the phosphate transfer reaction[78] (Fig. 5). The mechanism suggests that initial binding of Ado to the open active site of the enzyme induces a domain rotation around the di-glycine hinge ($Gly_{61}$-$Gly_{62}$) motif. $Arg_{69}$, located on the α-3 helix possibly facilitates such domain movement. This leads to a relatively closed positioning of the lid and places the second substrate (i.e., ATP) in a catalytically competent position, thereby allowing the active-site located $Asp_{299}$ to accept a proton from the 5'-group of the ribose of Ado resulting in direct nucleophilic attack on the terminal phosphate of ATP by an in-line $S_N2$ mechanism. Results further suggested that during the whole process, $Arg_{131}$ acts as the bidentate electrophile. First, it stabilises the resulting quinquivalent transition state by interacting with two negatively charged oxygen groups of the terminal phosphate of ATP and second, $Arg_{131}$ possibly helps in increasing the electrophilicity of the γ-phosphorus atom by withdrawing the negative charge of the oxygen atoms.

## Product-Mediated Enzyme Regulation

LdAdK, similar to AdK from most sources, is known to be inhibited by AMP and ADP, raising the possibility of product-mediated regulation.[59,61,79] It is well known that AMP is a

competitive inhibitor of the enzyme with respect to Ado and noncompetitive with respect to ATP. In contrast, ADP behaved as a noncompetitive inhibitor with respect to both Ado and ATP, with inhibition by ADP becoming uncompetitive at higher concentrations of ATP.[59] However, until recently, it was not known as to whether the same amino acids were involved in binding both Ado and AMP. Moreover, very little information with regard to ADP binding site were available. However, development of the $Asp_{16}$ mutant, defective in Ado binding, permitted investigations on the mechanism of AMP inhibition. In these studies it has been shown that although Ado and AMP occupy a nearly overlapping position resulting in apparent competition between the two, their mode of interaction with the enzyme are not exactly identical. Analysis suggested that $Arg_{131}$, which has been identified as the key residue involved in the phosphotransfer mechanism, plays an additional role in AMP binding, thereby acting as an effector for product-mediated enzyme regulation. This dual role of $Arg_{131}$ (both in catalysis as well as in regulation) has been further supported with the help of the AMP-docked structure of LdAdK.[65]

Apart from the AMP-mediated regulation, ADP also appears to regulate the activity of the enzyme. Although evidence in favour of this notion is still scanty and will require extensive investigations, the available in vitro results suggest that unlike AMP, the ADP-mediated regulatory mechanism involves the simultaneous participation of another chaperonic protein viz. cyclophilin (CyP). It has recently been demonstrated that LdAdK, which has an inherent tendency to form inactive soluble oligomers, could be disaggregated by a cyclophilin from *L. donovani* (LdCyP) in an isomerase-independent fashion, resulting in reactivation.[80] The reactivation of LdAdK could be achieved in vitro with a stoichiometric amount of LdCyP and under simulated in vivo condition.[81] While investigating the mechanism, it was discovered that ADP, generated during the AdK reaction, facilitates formation of these AdK aggregates, leading to its inactivation. Detailed analysis of the mechanism of reactivation suggested that LdCyP-induced reactivation occurs due to conformational reorientation of AdK in a manner that decreases the affinity of the enzyme for ADP resulting in disaggregation of the inactive oligomers to active monomers.[82] A mechanism of ADP-mediated regulation of LdAdK has also been proposed (Fig. 6).

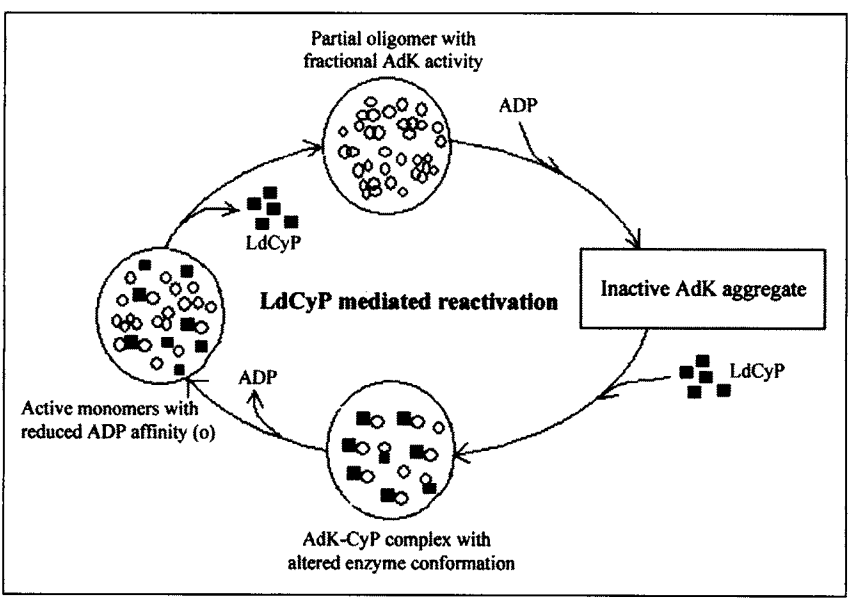


Figure 6. Schematic representation of cyclophilin mediated reactivation of adenosine kinase. Reprinted with permission from: Sen et al, Biochemistry 2006; 45:263-271; ©2006 American Chemical Society.[82]

Since, nucleotide-induced aggregation-disaggregation of enzymes forms the basis of enzyme regulation in many cases, the likelihood of this mechanism operating in *L. donovani* cannot be ruled out.[82]

Based on these observations, it has been possible to arrive at a point from where the "catalytic movie" of LdAdK during the progression of reaction can be speculated (Fig. 7). The self-explanatory cartoon pinpoints the likely conformational change that possibly occurs during the overall process. The ADP-induced aggregation of the enzyme, which may form a basis for enzyme regulation, has however been excluded from this proposed mechanism.

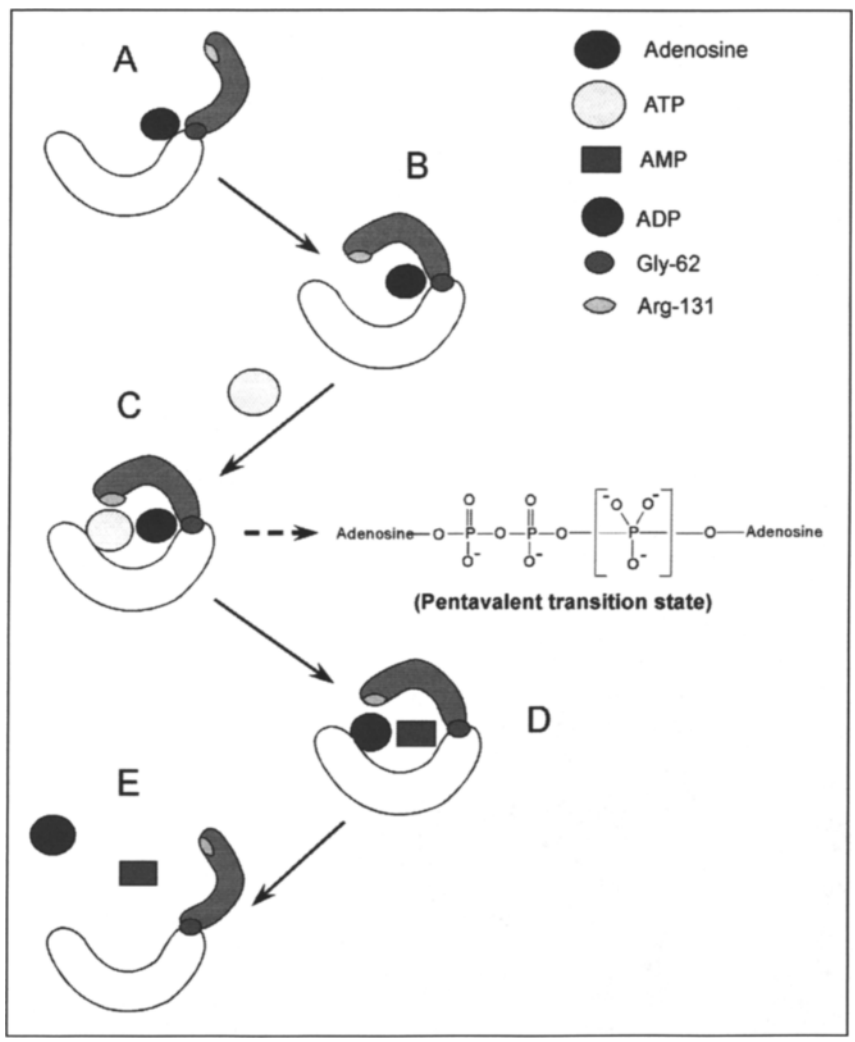


Figure 7. Cartoon representation of the proposed LdAdK catalysed reaction. A) Ado binds to the open active site of the enzyme followed by, B) a rotation of the small domain around the di-glycine hinge causing domain closure and recruitment of Arg-131 to the active site; C) Binding of ATP to the closed active site resulting in phosphate transfer via a pentavalent transition state; D) Formation of the products and, E) Subsequent product release.

## Conclusions and Perspectives

Extensive research over the last several years has identified several unique metabolic pathways obligatory to survival and multiplication of parasites. Of these prospective pathways, this chapter has tried to articulate the importance of the pathway responsible for the salvage of purine nucleotides of the purine auxotrophic parasitic protozoa, with special reference to AdK of *Leishmania.* Our interest in this pathway stemmed from the observation that LdAdK, being the gateway enzyme for adenosine nucleotide assimilation in *Leishmania,* shows stage-specific activity profile during morphogenic transformation of the parasite and the possibility of synthesizing subversive nucleoside analogs capable of selectively inhibiting the AdK-mediated phosphorylation reaction in *Leishmania* exists. Our aim has been to address two specific questions: (i) what structural features of this parasitic enzyme are potentially important for the process of phosphate transfer and substrate binding and (ii) how do these features relate to the transition state of catalysis and the overall reaction mechanism? To this end, homology modeling of LdAdK has allowed visualization of the active site of the enzyme and analyse the results of the mutagenesis experiments. Although the actual crystal structure of the protein is a must, the model would be useful in identifying additional sites for mutagenesis and conceptualising the results until the structure of LdAdK is determined experimentally. An additional point of significance of these findings is that, by understanding the structural requirements of product binding, one can certainly conceive of strategies for designing inhibitors capable of interacting with the product binding sites. Therefore, studies directed towards exploiting LdAdK as the target for designing structure-based inhibitor or other enzymes of the purine salvage pathway might prove rewarding.

### *Acknowledgement*

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