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Commentary

Molecular genetics of nucleoside transporters in *Leishmania* and African trypanosomes

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Abstract

Nucleoside transporters play central roles in the biochemistry of parasitic protozoa such as *Leishmania* and African trypanosomes, because these parasites cannot synthesize purines *de novo* and are absolutely reliant upon purine salvage from their hosts. Furthermore, nucleoside transporters are important to the pharmacology of these significant human pathogens, because they mediate the uptake of purine analogs, as well as some non-purine drugs, that are selectively cytotoxic to the parasites. Recent advances in molecular biology and genomics have allowed the cloning and functional expression of several nucleoside transporter genes from *Leishmania donovani* and *Trypanosoma brucei*, providing molecular reagents for a detailed functional examination of these permeases and their role in the delivery of nutrients and drugs to the parasites. Furthermore, the molecular basis of drug-resistant mutants that are deficient in nucleoside transport functions can now be fathomed.

Keywords: Nucleoside transport; *Leishmania;* Trypanosomes; Purine salvage; Purine analogs; Drug resistance

1. Introduction

Leishmania species and *Trypanosoma brucei* are parasitic protozoa of the order Kinetoplastida that cause leishmaniasis and African sleeping sickness [1]. Both diseases are prevalent in various topical regions of the globe and are prominent causes of illness and death in endemic areas. Treatment and control of these important tropical diseases are compromised by the toxicity and expense of current drugs, the slow development of novel therapies, and the increasing emergence of drug-resistant strains [2]. While the drugs that are now widely used were discovered empirically, the identification of novel anti-parasitic compounds is likely to be advanced considerably by knowledge of biochemical pathways that differ between the parasite and the host and that may be targeted for selective toxicity against the pathogen.

The transport of nucleosides and nucleobases across the plasma membrane of parasitic protozoa has elicited considerable attention due to the central role of purine metabolism in these microorganisms. Unlike their vertebrate hosts, all parasitic protozoa examined to date are unable to synthesize purines *de novo* and, hence, rely absolutely upon the salvage of these compounds from their hosts [3,4]. The first step in these salvage pathways is the transport of the preformed purines across the surface membrane of the parasite, underscoring the importance of nucleoside and nucleobase permeases for parasite nutrition. Moreover, the permeases involved in purine salvage also mediate the uptake of pyrazolopyrimidines, isomers of naturally occurring purines that are selectively cytotoxic to the parasites [4]. Several of these compounds are currently being tested or are in use against parasitic infections in the field [5,6]. The selective action of these compounds, including allopurinol, allopurinol riboside, 4-thiopurinol, 4-thiopurinol riboside, and formycin B, arises from the distinct substrate specificities of several parasite salvage enzymes [4], notably hypoxanthine guanine phosphoribosyltransferase and adenylosuccinate synthetase. These enzymes allow the incorporation of the pyrazolopyrimidines into the nucleoside triphosphate pool of the parasite, whereas the corresponding interconversions do not occur efficiently in mammalian cells. Hence, while the parasite permeases are not the selective agents themselves, they are responsible for drug delivery to the target. Furthermore, one clearly demonstrated mechanism of resistance to pyrazolopyrimidines involves mutations that impair

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Abbreviations: ORF, open reading frame; and TMD, transmembrane domain.

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the uptake of nucleosides [7,8], thus preventing the drug from reaching its target.

2. Studies on nucleoside and nucleobase uptake by intact parasites

Studies by Ullman and colleagues over a decade ago [7,8] established that *Leishmania donovani*, the agent of fatal visceral leishmaniasis, possesses two distinct nucleoside transporters with non-overlapping substrate specificities, one of which mediates the uptake of adenosine and the pyrimidine nucleosides and the other of which imports guanosine and inosine (Fig. 1). Mutant lines could be generated that were deficient in either one of these activities, supporting the assignment of the two distinct transport functions. Thus, the TUBA5 cell line, selected in the presence of the cytotoxic adenosine analog tubercidin, was deficient in the uptake of tubercidin, adenosine, and the pyrimidine nucleosides, while the FBD5 cell line, selected in the presence of the inosine analog formycin B, was deficient in the uptake of formycin B, guanosine, and inosine. These substrate specificities were confirmed by the measurement of uptake of radiolabeled nucleosides by intact cells and by competition with unlabeled substrates. Furthermore, these permeases had K_m values in the submicromolar range for adenosine and inosine, revealing that the parasite uptake systems have 100- to 1000-fold lower K_m values than typical mammalian nucleoside transporters [7]. While this work was carried out on the promastigote stage of the parasite life cycle that lives within the sandfly vector responsible for transmission of the infection between vertebrate hosts, recent studies on the amastigotes that live within the phagolysosomal vesicles of the vertebrate macrophages have revealed another amastigote-specific transport activity specific for the purine nucleosides adenosine, guanosine, and inosine [9].

Related studies by Carter and Fairlamb [12] identified two distinct nucleoside transport activities in African trypanosomes (Fig. 1). The P1 activity mediates the uptake of adenosine, guanosine, and inosine and is present in both the procyclic forms that live in the tsetse fly vector and the bloodstream forms that live in the mammalian host. In contrast, the P2 activity transports both adenosine and the nucleobase adenine and is expressed only in the bloodstream parasites. Remarkably, the P2 transporter is also responsible for the uptake of the non-purine drugs melarsoprol [12] and pentamidine [13] that are used to treat African sleeping sickness, and a drug-resistant parasite line has been established that is also deficient in P2 transport activity. A common structural motif present in the 6-aminopurines adenosine and adenine and in melarsen oxide and pentamidine has been suggested as the molecular recognition element that mediates interaction of each compound with the P2 transporter [14].

Detailed kinetic studies by Jarvis and colleagues [15] on nucleoside uptake by procyclic trypanosomes have defined the properties of a high-affinity P1 type purine nucleoside transporter. This permease induces an influx of protons that accompany adenosine import, is sensitive to reduction of the transmembrane proton gradient, and is inhibited by an increase in pH, strongly suggesting that the transporter is an active proton symporter. This work underscores the efficiency with which the parasite transporters can compete with the host for essential purines and also their efficacy as agents for delivering cytotoxic purine analogs to the parasite. Similar studies on nucleoside uptake by bloodstream trypanosomes have confirmed the existence of P1 and P2 activities, compared their kinetic parameters and substrate recognition profiles, and established their likely function as proton symporters [16]. Furthermore, elegant kinetic studies by de Koning and Jarvis have identified two nucleobase transport activities, H1 and H2, in bloodstream trypanosomes [17], and a purine nucleobase transporter [18] and a uracil transporter [19] in procyclic trypanosomes, all of which appear to be proton symporters. This work highlights the multiplicity of nucleoside and nucleobase transport systems available to these unicellular organisms for the salvage of these crucial nutrients.

Transport activities for another potential purine source, *S*-adenosylmethionine, have been identified in both *L. donovani* [10] and *T. brucei* [11]. These permeases may supplement the purines delivered to the parasites by other transport systems and may also provide vehicles for delivery of drugs that are structural analogs of *S*-adenosylmethionine [20].

3. Cloning and functional expression of *Leishmania* nucleoside transporters

The existence of the mutant tubercidin-resistant TUBA5 and formycin B-resistant FBD5 lines of *L. donovani*, deficient in adenosine/pyrimidine nucleoside and guanosine/inosine transport, respectively, has allowed the cloning of the corresponding transporter genes by functional complementation [21,22]. In these experiments, a genomic cosmid expression library [23] was transfected into each transport-deficient mutant cell line, and approximately a thousand clonal transfectants were screened for the reacquisition of drug sensitivity that should accompany expression of the wild-type transporter.

A cosmid clone that conferred tubercidin sensitivity upon the TUBA5 cells contained two closely linked ORFs that coded for proteins that differed by only 6 amino acids, designated LdNT1.1 and LdNT1.2. Each of these proteins restored drug sensitivity and adenosine transport activity to the mutant cells [22]. The amino acid sequences of both proteins were 33% identical to the human equilibrative nucleoside transporter hENT1 [24], and, like the human protein, they predicted the existence of 11 TMDs (Fig. 2). The functional properties of each permease were deter-

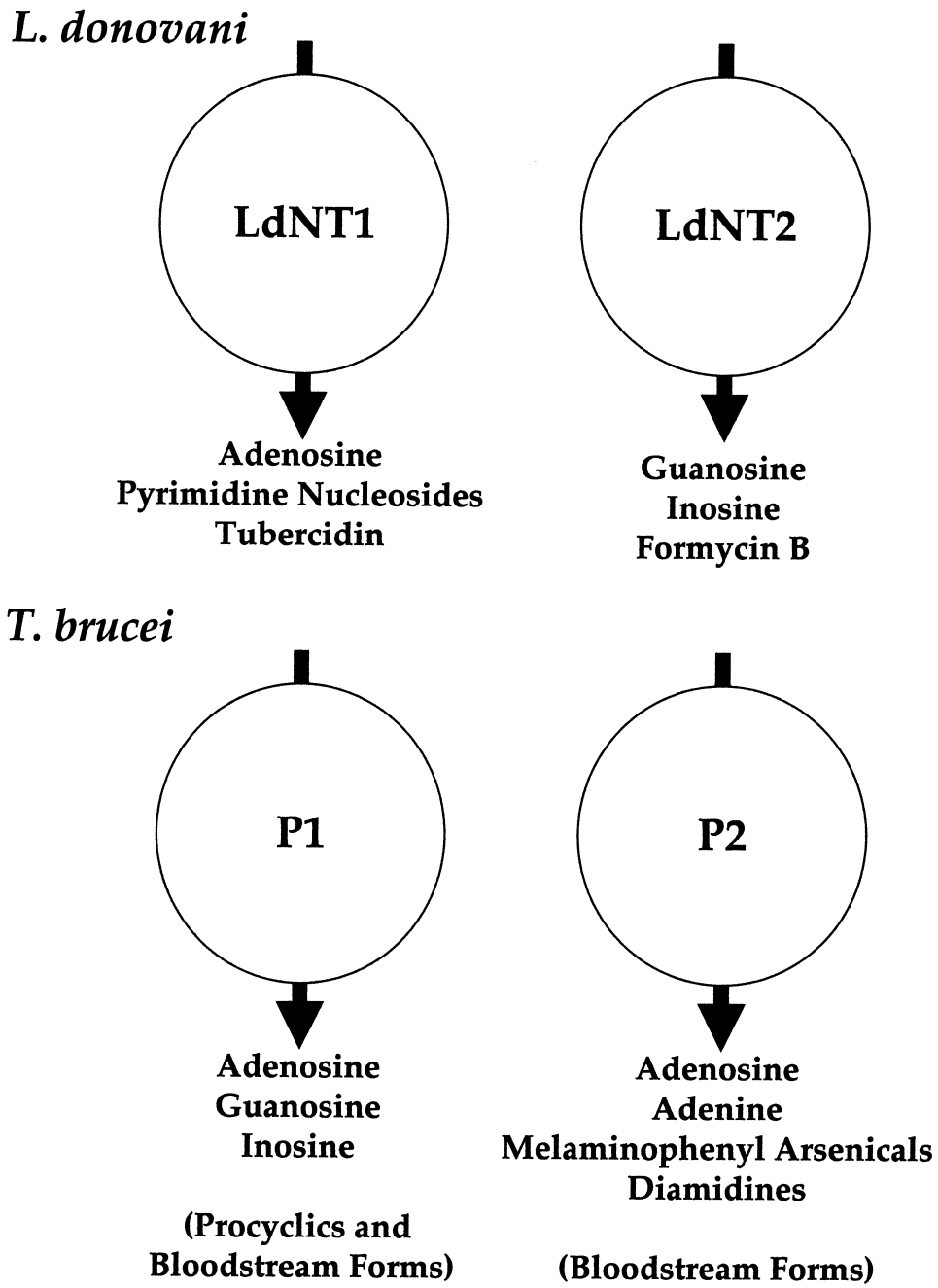

Fig. 1. Nucleoside transport activities in *L. donovani* (top) and *T. brucei* (bottom). In *L. donovani*, the LdNT1 transport activity mediates the uptake of adenosine, pyrimidine nucleosides, and the toxic adenosine analog tubercidin and is accomplished by two closely related transporters, LdNT1.1 and LdNT1.2. The LdNT2 permease transports guanosine, inosine, and the toxic inosine analog formycin B. In *T. brucei*, the P1 activity imports the purine nucleosides adenosine, guanosine, and inosine. This activity is present in both procyclic and bloodstream forms and is apparently achieved by a multiplicity of permeases. The P2 transporter takes up adenosine, the purine base adenine, melaminophenyl arsenical drugs such as melarsoprol, and diamidines such as pentamidine and is expressed only in bloodstream forms of the parasite. There are also less well characterized transport activities, such as a purine nucleoside transporter from the intracellular amastigote stage of the *L. donovani* life cycle [9] and *S*-adenosylmethionine transporters from both *L. donovani* [10] and *T. brucei* [11].

mined by expressing the corresponding ORF in the TUBA5 cell line. Both permeases mediated the uptake of radiolabeled adenosine and pyrimidine nucleosides, but the LdNT1.1 protein exhibited a higher affinity for substrates than LdNT1.2 (K_m values for adenosine ~0.2 and ~5 μM, respectively). In addition, both permeases were expressed in *Xenopus* oocytes, where they exhibited transport properties similar to those observed in *L. donovani.* These structural and functional studies reveal that the parasites express nucleoside transporters that are related in sequence to the mammalian equilibrative transporters but that are adapted to function with much higher affinity and probably as active transporters [7], consistent with the need of the parasite to compete with the host for purines.

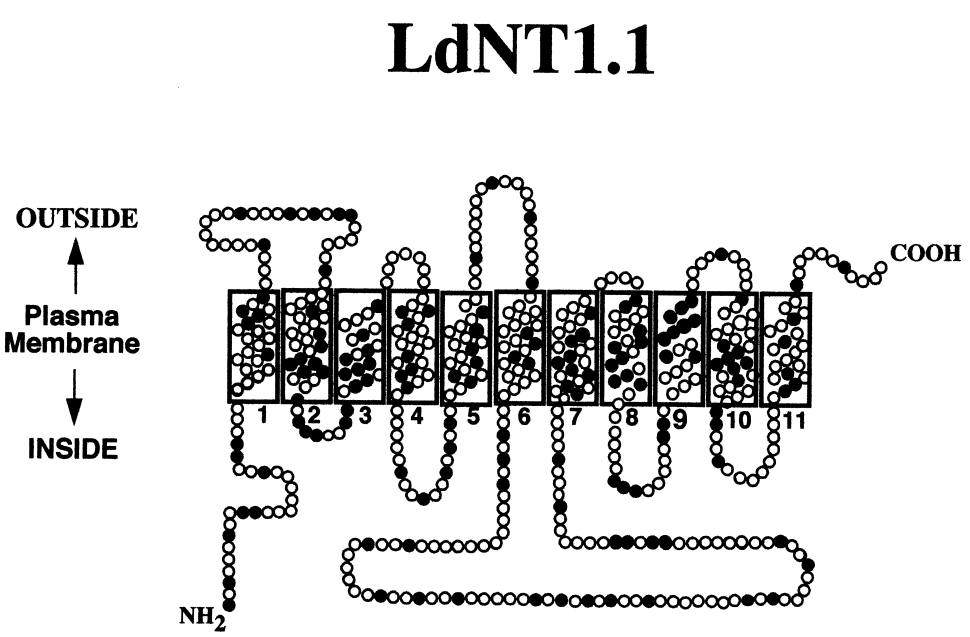

Fig. 2. Topology model for the LdNT1.1 nucleoside transporter from *L. donovani*. Circles represent individual amino acids, and black circles indicate amino acids that are identical to those in the human equilibrative nucleoside transporter hENT1. Rectangles designate predicted [25] transmembrane segments.

The availability of the cloned *LdNT1.1* and *LdNT1.2* transporter genes has allowed the examination of the molecular nature of the mutations leading to transport deficiency in the TUBA5 line. The genes are not rearranged, truncated, or deleted nor is the mRNA level reduced in the mutant cell line [22]. However, cloning of the *LdNT1.1* and *LdNT1.2* genes from TUBA5 cells revealed two alleles of *LdNT1.1*, each with a distinct point mutation in the ORF, whereas the *LdNT1.2* genes were wild type [26]. The existence of individual debilitating mutations suggests that the forward genetic approach used to generate the TUBA5 cell line may be useful to define structure–function relationships for this permease. The fact that the *LdNT1.2* genes were wild type in the transport-deficient TUBA5 cells might be explained by the fact that the level of LdNT1.2 mRNA is extremely low in wild-type cells, raising the possibility that these genes contribute little to the transport phenotype.

The *LdNT2* guanosine/inosine transporter gene was cloned by expression in FBD5 transport-deficient cells by screening for restoration of sensitivity to the inosine analog formycin B [21]. *LdNT2* is a single copy gene that predicts a protein with 44% amino acid identity to the LdNT1.1 protein. Expression of the LdNT2 protein in FBD5 cells restored inosine and guanosine transport activities with K_m values of 0.3 and 1.7 μM, respectively, and with the substrate inhibition profile predicted for this permease from uptake studies in wild-type cells [7]. Similar to *LdNT1.1*, *LdNT2* can be functionally expressed in *Xenopus* oocytes, and the mutant FBD5 line does not contain any apparent gene rearrangements or reduction in mRNA level.

4. Molecular genetics of nucleoside transporters in African trypanosomes

The P2 transporter gene of *T. brucei*, responsible for the uptake of adenosine, adenine, and the drugs melarsoprol and pentamidine, was cloned by Kaminsky and colleagues [27] in elegant studies involving functional expression and selection for adenosine protrophy in the *ade2* mutant of *Saccharomyces cerevisiae*. Yeast does not normally transport adenosine, but expression of the P2 transporter in the purine biogenesis-defective *ade2* line allowed rescue of this mutant by growth in adenosine as the sole purine source. The *TbAT1* ORF obtained from this selection coded for a protein that conferred transport of [^{3}H]adenosine in yeast; it was inhibited by unlabeled adenosine or adenine but not by other naturally occurring nucleosides or nucleobases, and was also inhibited by the anti-trypanosomal drugs melarsoprol and pentamidine. These results confirm that TbAT1 is the P2 transporter and conclusively demonstrate that it mediates uptake of non-purine drugs as well as purines. The TbAT1 protein was 30% identical in sequence to LdNT1.1, although the TbAT1 sequence predicts 10 TMDs instead of 11. Furthermore, a melarsoprol-resistant line of *T. brucei*, STIB777R, contained a *TbAT1* gene with ten nucleotide differences, six of which cause amino acid substitutions, compared with the transport-competent line from which it was derived.

A gene encoding a P1 type transporter from *T. brucei* has also been cloned by homology to the *L. donovani* LdNT1.1 transporter [28]. Expression of this TbNT2 permease in *Xenopus* oocytes stimulates uptake of radiolabeled adenosine, inosine, and guanosine. The TbNT2 protein has sig-

nificant sequence identity to TbAT1, LdNT1.1, and LdNT2 and predicts 11 TMDs, similar to LdNT1.1 and hENT1. Notably, TbNT2 mRNA is expressed exclusively in bloodstream trypanosomes, despite the fact that P1 activity is present in both the bloodstream and procyclic parasites (Fig. 1) [12,15]. Consequently, there must be at least one other P1 type transporter that is expressed in procyclic trypanosomes. Furthermore, genomic Southern blots probed with the *TbNT2* ORF revealed the presence of a family of related genes [28], and studies in progress[1] show that *TbNT2* is part of a genomic cluster of six closely related genes. Functional characterization of each member of this gene family should reveal whether there are any biochemical distinctions that might explain the existence of the discrete transporter isoforms.

5. Conclusions and perspectives

The cloning and functional expression of nucleoside transporters have provided the molecular reagents for probing the structure and mechanisms of action of this important family of permeases, a process that is only now beginning. One issue of immediate importance is to determine how many other nucleoside transporter genes may exist in each of these parasites so that we can arrive at a global picture of how these nutrients are acquired by each organism. In the case of *L. donovani*, it is likely that LdNT1.1, LdNT1.2, and LdNT2 are the only nucleoside transporters expressed in the promastigote stage of the life cycle, since the TUBA5/FBD5 double mutant is completely deficient in uptake of nucleosides [7]. However, a recent report of a unique nucleoside transport activity present in amastigotes of *L. donovani* [9] suggests that another amastigote-specific permease may exist.

For *T. brucei*, the only nucleoside permease genes that have been cloned and well characterized to date are *TbAT1* and *TbNT2*. However, the existence of six genes within the *TbNT2* cluster reveals that other isoforms are likely to be expressed, and these permeases are now being characterized regarding their biochemical function and expression during the parasite life cycle. Furthermore, the observation that TbNT2 is a bloodstream form-specific P1 transporter indicates that there must be at least one additional P1 type transporter that is expressed in procyclic-form parasites. Our laboratory has identified another gene that is related in sequence to the known nucleoside transporters and whose mRNA is expressed in procyclic parasites; the encoded permease is now being characterized. The existence of this multiplicity of nucleoside transporters in *T. brucei* was not anticipated on the basis of uptake studies on intact parasites, and this fact underscores the importance of molecular and genomic approaches to understanding nucleoside uptake in these parasites. What is required now is a detailed analysis of all family members to determine the specific biochemical and physiological functions that each member subserves. The existence of strongly developmentally regulated transporter genes suggests that distinct permease isoforms may be required to allow procyclic and bloodstream forms to adapt ideally to their distinct physiological environments.

The fact that both *Leishmania* and trypanosomes possess so many pathways for uptake of purines as either the nucleosides or nucleobases implies that these permeases would be very difficult to target with inhibitors for selective anti-parasite drug development. Nonetheless these transporters will continue to be of pharmacological importance, as they provide the routes for delivery of selectively cytotoxic purine analogs as well as some other non-nucleoside drugs. Furthermore, it is clear that mutations in the transporter genes can be an effective mechanism for the generation of drug-resistant parasites, a problem of increasing importance in the field [2].

Another significant frontier in research on nucleoside transporters will be to determine how the structures of these polytopic membrane proteins confer their functional properties. Structural studies on membrane proteins have typically been extremely challenging due to the low levels of their expression and the difficulty in purifying and crystallizing them in native form. Nonetheless, recent advances in structural determination of both bacterial [29] and eukaryotic [30] membrane proteins raise the hope that it will eventually be possible to obtain high resolution structural information with greater facility than is currently the case. Furthermore, a variety of chemical approaches [31,32] have been advanced in recent years that should allow us to probe significant features of permease structure. Thus, while several topologies have been suggested for both mammalian [24] and parasite [21,22,27] nucleoside transporters, these models remain to be evaluated experimentally. Furthermore, the nature of the 'permeation pathway' that permits passage of the organic substrate through the hydrophobic barrier is undefined for these transporters, as are the determinants of substrate specificity. A significant effort employing biochemical, physical, and genetic approaches is likely to be required to elucidate these features of transporter function.

One notable distinction between the mammalian and parasite transporters in this family is that the former are equilibrative permeases that do not concentrate their substrates, whereas the latter are likely to be active transporters that utilize the strong proton electrochemical gradient across the parasite plasma membrane [33,34] to concentrate these important nutrients within the parasite. The existence of electrogenic transport processes raises the possibility of applying electrophysiological methods [35] to study the mechanism of action of these transporters, including the identification of co-transported ions, the stoichiometry of co-transport, and the identification of partial steps in the reaction cycle.

[1] Sanchez M. Manuscript in preparation. Cited with permission.

One final topic of interest concerns the regulation of nucleoside transporter expression in *Leishmania* and trypanosomes. Some years ago, Gottlieb [36] observed that the activity of 3′-nucleotidase, a membrane-bound enzyme whose apparent role in purine salvage is to remove the 3′-phosphate groups from extracellular nucleoside monophosphates to provide substrates for the nucleoside transporters, is dramatically up-regulated by starvation for purines in the trypanosomatid *Crithidia luciliae.* Subsequent studies by Gottlieb and coworkers [37,38] and Gero and coworkers [39] revealed that nucleoside and nucleobase uptake was similarly up-regulated in *C. luciliae*, 7- and 100-fold, respectively, by starvation for purines. Furthermore, in *L. donovani* the transport of adenosine via the LdNT1.1 transporter is up-regulated by withdrawal of its substrate adenosine but not by withdrawal of other purines that are not substrates for LdNT1.1 [40]. Together, these results reveal an adaptive mechanism that increases the ability of these parasites to salvage purines more efficiently as their extracellular concentrations decline. The observation that the mRNA levels for nucleoside transporters in *C. luciliae* were not affected by purine starvation, whereas the level of transport activity was dramatically stimulated [41], suggests that these regulatory mechanisms are likely to operate at the post-transcriptional and even the post-mRNA level. The challenge for the future will be to define the molecular mechanisms for this regulatory process, specifically whether it operates at the level of transporter protein turnover, chemical modification of the permease, or mRNA translation.

The expression of some nucleoside transporters is strictly regulated during the parasite life cycle, including TbAT1 and TbNT2, which are expressed exclusively in bloodstream-form trypanosomes [27,28]. Many genes in *Leishmania* and trypanosomes are regulated post-transcriptionally, often at the level of mRNA stability [42]. It will be important to determine the level of regulation for nucleoside transporter genes and the molecular determinants of this stage-specific gene regulation. Hence, nucleoside transporters offer windows into many aspects of the biology of these medically important parasites.

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