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Molecular Interactions Underlying the Unusually High Adenosine Affinity of a Novel *Trypanosoma brucei* Nucleoside Transporter[S]

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ABSTRACT

Trypanosoma brucei encodes a relatively high number of genes of the equilibrative nucleoside transporter (ENT) family. We report here the cloning and in-depth characterization of one *T. brucei brucei* ENT member, TbNT9/AT-D. This transporter was expressed in *Saccharomyces cerevisiae* and displayed a uniquely high affinity for adenosine (K_m = 0.068 ± 0.013 μM), as well as broader selectivity for other purine nucleosides in the low micromolar range, but was not inhibited by nucleobases or pyrimidines. This selectivity profile is consistent with the P1 transport activity observed previously in procyclic and long-slender bloodstream *T. brucei*, apart from the 40-fold higher affinity for adenosine than for inosine. We found that, like the previously investigated P1 activity of long/slender bloodstream trypanosomes, the 3′-hydroxy, 5′-hydroxy, N3, and N7 functional groups contribute to transporter binding. In addition, we show that the 6-position amine group of adenosine, but not the inosine 6-keto group, makes a major contribution to binding (ΔG^0 = 12 kJ/mol), explaining the different K_m values of the purine nucleosides. We further found that P1 activity in procyclic and long-slender trypanosomes is pharmacologically distinct, and we identified the main gene encoding this activity in procyclic cells as *NT10/AT-B*. The presence of multiple P1-type nucleoside transport activities in *T. brucei brucei* facilitates the development of nucleoside-based treatments for African trypanosomiasis and would delay the onset of uptake-related drug resistance to such therapy. We show that both TbNT9/AT-D and NT10/AT-B transport a range of potentially therapeutic nucleoside analogs.

Purine salvage is an essential function for *Trypanosoma* spp., in that they are unable to synthesize the purine ring de novo (Berens et al., 1995). Consequently, the *Trypanosoma brucei brucei* nucleoside transporters are of both physiological and pharmacological interest, and several purine transport activities and transporter genes have been identified (de Koning et al., 2005). Most of the pharmacological interest has centered on the TbAT1/P2 aminopurine transporter because of its unusual substrate profile (high affinity for both adenine and adenosine but not for oxypurines or pyrimidines) and its involvement in the accumulation of the major trypanocidal drugs by the parasite (Carter and Fairlamb, 1993; de Koning, 2001). The detailed understanding of the molecular interactions involved in substrate-transporter binding allowed the synthesis of novel trypanocides specifically targeted to the trypanosome (Tye et al., 1998; Soulère et al., 2000; Baliani et al., 2005). One major drawback of this strategy, however, is that loss of TbAT1/P2 activity is already associated with resistance to the current front-line drugs against African trypanosomiasis, better known as sleeping sickness (Delespaux and de Koning, 2007; Lüscher et al., 2007).

In contrast, the P1 transport activity has received little attention, although it has been clear for some time that the *T. brucei brucei* genome contains multiple equilibrative nucleoside transporter (ENT) family genes encoding P1-type transporters (Sanchez et al., 1999, 2002, 2004), and the completion

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ABBREVIATIONS: ENT, equilibrative nucleoside transporter; AT, adenosine transporter; RT, reverse transcriptase; ORF, open reading frame; LS, long slender; SS, short stumpy; PC, procyclic; kb, kilobase(s).

of the *T. brucei brucei* genome revealed several additional ENT genes. The simultaneous expression of several related purine nucleoside transporters would be of interest because it would delay the onset of resistance to purine antimetabolites accumulated through multiple transport proteins. Bloodstream trypanosomes are already known to express at least two high-affinity hypoxanthine transporters (de Koning and Jarvis, 1997) that seem to prevent the induction of resistance to the hypoxanthine analog allopurinol (Natto et al., 2005). Second, the presence of multiple nucleoside transporters in the parasite would allow the efficient uptake of a wider spectrum of purine antimetabolites, especially because nutrient transporters of kinetoplastids, including *Trypanosoma* spp. and *Leishmania* spp., display much higher affinity for their substrates and are usually energy-dependent and monodirectional (for review, see Lüscher et al., 2007). The pharmacological potential of the P1 activity was very recently demonstrated by Geiser et al. (2005), who showed that 3′-deoxyadenosine (cordycepin) is an extremely potent trypanocide and that its uptake is mediated by both the P1 and P2 activities.

In the current article, we report for the first time that multiple, pharmacologically distinct but very similar P1-type transport activities can be identified in the various *T. brucei brucei* life cycle stages—distinguished by sensitivity to dipyridamole and 5′-deoxyadenosine. Moreover, we report the cloning of two P1-type transporters from *T. brucei brucei* and characterize them while expressed in the yeast *Saccharomyces cerevisiae*. The cloning and partial characterization of one, *NT10/AT-B*, has previously been reported by Sanchez et al. (2004), and we report here a fuller characterization in yeast as well as a first demonstration of this transport activity in *T. brucei brucei* as the main P1 transporter in procyclic trypanosomes. We also demonstrate that this transporter is capable of mediating uptake of multiple antimetabolites.

Most notably, we cloned a new P1-type transporter, NT9/AT-D, that showed a uniquely high affinity for adenosine and 2′-deoxyadenosine when expressed in yeast. We present a model explaining this exceptional feature on the basis of the transporter's interactions with its substrate.

Materials and Methods

Materials. [2-^{3}H]Adenosine (0.92 TBq/mmol), [5,6-^{3}H]uracil (1.78 TBq/mmol), and [8-^{3}H]-hypoxanthine (1.18 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). [2,8-^{3}H]-Adenine (1.19 TBq/mmol) was bought from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]7-Deazaadenosine (11 Ci/mmol), [^{3}H]3-deazaadenosine (12.1 Ci/mmol), and [2, 8-^{3}H]inosine (1.23 TBq/mmol) were obtained from Moravek Biochemicals (Brea, CA). Standard chemicals, nucleosides, and nucleobases were obtained from Sigma (St. Louis, MO) unless otherwise stated. 6-Chloropurine riboside and 7-deaza-2′-deoxyadenosine were from TriLink BioTechnologies (San Diego, CA); tubercidin (7-deazaadenosine) and cordycepin (3′-deoxyadenosine) were from Fluka (Buchs, Switzerland); cytidine and adenine-9-β-D-arabinofuranoside (AraA) were from ICN; 1-Deazaadenosine was a generous gift from Prof. G. J. Koomen (University of Amsterdam, The Netherlands).

Organisms. *T. brucei brucei* of the pleomorphic strain TREU927 were used throughout this study. The *S. cerevisiae* strain MG887-1 (*fcy2*−) used in this project was auxotrophic for uracil and does not possess a functional purine transporter (Gillissen et al., 2000). JM109 competent *Escherichia coli* were obtained from Promega (Madison, WI).

Cloning of NT9/AT-D and NT10/AT-B. Several ENT homologues were identified from TBLASTN searches of the Wellcome Trust Sanger Institute sequence databases (http://www.sanger.ac.uk/projects/T_brucei). The cloning and characterization of one of these was previously reported as *TbNBT1* (Burchmore et al., 2003). Two others were designated *AT-B* and *AT-D* and the following PCR primers from regions flanking the ORF were designed: *AT-B*: forward, 5′-CTTCACCATCTAGCTGAGTCC-3′; reverse, 5′-TTACCTCCTCTTAGGGACAG-3′; AT-D: forward 5′-GATGCTCGGTTTTTATTCAG-3′; reverse, 5′-ATGTCACACTGGTTATTTCAGC-3′. Genomic DNA was isolated from procyclic *T. brucei brucei* s927 grown in SDM79 culture medium supplemented with 10% fetal calf serum (Brun and Schönenberger, 1979), following standard protocols. The ORFs of *AT-B* and *AT-D* were amplified by PCR using *Pfu* proofreading DNA polymerase (Stratagene, La Jolla, CA), cloned into the vector pGEM-Teasy (Promega) and amplified in *E. coli* JM109. For both genes, the cloned products of two independent PCR reactions were sequenced on both strands (MWG Biotech); the two independent clones were found to be identical. The cloning and partial characterization of *AT-B* as *TbNT10* has since also been reported by Sanchez et al. (2004).

Expression and Characterization of *T. brucei brucei* Nucleoside Transporters in *S. cerevisiae*. The complete *AT-B* or *AT-D* ORF was excised from pGEM-Teasy using NotI and subcloned into the NotI site of the yeast expression vector pDR195 (Rentsch et al., 1995), which was transformed into a uracil auxotrophic *fcy2*− strain of *S. cerevisiae*, MG887-1, and selected on uracil-free medium, as described previously (Burchmore et al., 2003). Transformed yeast was plated onto yeast nitrogen base without ammonium salts and amino acids, supplemented with 4 mM hypoxanthine or 1 mM adenosine as the sole nitrogen source. Purine transport in (transformed) yeast was performed with cells grown in complete minimal medium without uracil, to a density of 1.5 to 2 OD_{600} units, as described previously for *S. cerevisiae* (Burchmore et al., 2003) and *T. brucei brucei* (Wallace et al., 2002). The method used an incubation of ~10^7 cells with an equal volume of buffer containing radiolabeled permeant and (where indicated) an inhibitor at appropriate concentrations. Incubations are stopped by the addition of excess volume ice-cold unlabeled permeant at saturating concentrations and immediate centrifugation, separating the cells from radiolabel by pelleting them under an oil layer. Nonspecific association of radiolabel (zero uptake) was determined in the presence of 1 mM unlabeled permeant at 0°C and subtracted to yield mediated uptake.

Isolation and Staining of Short-Stumpy Bloodstream Forms. Adult female mice (ICR Swiss strain) were immunocompromised with cyclophosphamide (200 mg/kg) by intraperitoneal injection. After 24 h, mice were inoculated i.p. with ~1×10^6 of *T. brucei* strain 927 bloodstream forms. The blood was collected from the mice after 9 days. Using air-dried blood smear slides for routine Giemsa staining and NAD diaphorase assay, the levels of stumpy form trypanosomes were assessed microscopically. The remainder of the blood was centrifuged at 2500*g* for 15 min and trypanosomes from the buffy coat layer were purified by DE52 chromatography as described previously (Wallace et al., 2002).

NAD diaphorase assay was performed to confirm the presence of stumpy forms, which react positively, whereas long-slender forms do not (Vickerman, 1985). In brief, reaction solutions were made fresh just before use containing 4 mg of NADH disodium hydrate (Sigma-Aldrich), 0.8 ml of 0.1 M phosphate buffer, pH 7.3, and 1.2 ml of water, and thin air-dried blood smears were prepared. The slides were fixed at 4°C for exactly 5 min with 0.1 M cacodylate buffer, pH 7.2, containing 2.5% glutaraldehyde, and were then rinsed quickly in distilled water. Slides were incubated for 1 h with the reaction solutions (with and without NADH) before being mounted in glycerol and viewed under oil at 1000-fold magnification phase contrast mi-

croscopy. *T. brucei brucei* procyclic forms were used as positive control in this assay.

RT PCR of mRNA Isolated from Different Life Cycle Stages of *T. brucei*. Parasites from different life cycle stages of *T. brucei* [1) procyclic from a 2-day old culture of *T. brucei* strain 427 in SDM79/10% FCS; 2) a mid-log culture of long-slender bloodstream forms of *T. brucei* strain 427 in HMI-9/20% FCS (Invitrogen, Carlsbad, CA); 3) long-slender or short-stumpy bloodstream forms of *T. brucei* strain 927 isolated from infected mouse blood on a Whatman DE52 column] were harvested by centrifugation at 1000*g* for 3 min at 4°C and resuspended at 1×10^7 cells/ml. 1 ml of Tri reagent (Sigma) was added to 1 ml of cell suspension and mixed gently, incubated for 5 min at room temperature, and extracted with RNase-free chloroform (Sigma) and with isopropanol. The RNA was collected by centrifugation (13,000*g* for 15 min at 4°C) and washed with 500 μl of 75% ethanol. The pellet was resuspended in 30 μl of diethylpyrocarbonate-treated water and incubated at 55°C for 10 min. RNA samples were stored at −70°C for experimental use.

First-strand cDNA synthesis was performed using a SuperScript II reverse transcriptase (RT) (Invitrogen) according to the manufacturer's instructions. Control tubes were treated exactly in the same way but without adding the SuperScript II RT enzyme. Samples of the resulting cDNA and control samples were stored at −20°C until used in RT-PCR with primers to amplify *AT-B*, *AT-D*, and *TbNBT1*. Purity and integrity of the RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Data Analysis. The Prism 3 software package (GraphPad, San Diego, CA) was used to calculate the kinetic parameters, given as mean and S.E, using nonlinear regression. K_i inhibition constants and Gibbs free energy of interaction ΔG^0 were calculated as described previously (de Koning and Jarvis, 1999; Wallace et al., 2002). Errors given in tables and shown as bars in graphs are standard errors. In transport experiments, slopes were calculated by linear regression and determined to be significantly different from 0 or not using an *F* test calculated by the Prism 3 software.

Results

Molecular Cloning of Additional Members of the ENT Family from *T. brucei brucei*. TBLASTN searches, using the amino acid sequence of TbAT1, of genomic DNA sequence databases assembled at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/T_brucei/) yielded several unknown ORFs that aligned with known ENT family transporters *TbNT2* (Sanchez et al., 1999) and *TbAT1* (Mäser et al., 1999). These ORFs were provisionally named *AT-A* through *AT-E*. *AT-B* and *AT-D* seem to belong to a phylogenetic subgroup with the P1-type nucleoside transporters, *TbNT2* through *TbNT7*, and purine nucleoside transporters from *Crithidia fasciculata* and *Leishmania donovani* (de Koning et al., 2005). Hydropathy plots (using TMPRED, http://www.ch.embnet.org/software/TMPRED_form.html) suggested structures with 11 transmembrane domains and a large intracellular loop, consistent with the general ENT topology (Sankar et al., 2002). The amino acid sequences of *AT-B* and *AT-D* are displayed alongside *TbNT2* and *TbAT1* in Fig. 1 of the supplementary material available on-line.

AT-B has since been independently reported as *NT10* by Sanchez et al. (2004), and the AT-D sequence has also been identified as a potential ENT family homolog by Landfear et al. (2004) and named *NT9*. We will from here on refer to them as *NT9/AT-D* and *NT10/AT-B*, respectively. The two transporters were amplified by PCR and cloned into the vector pGEM-T Easy (Promega) as described above under *Cloning of NT9/AT-D and NT10/AT-B*, and subcloned into the yeast expression vector pDR195 as described previously (Burchmore et al., 2003). Orientation and integrity of the construct was verified using an appropriate series of restriction digests, after which the constructs were transformed into the *Saccharomyces cerevisiae* strain MG887.1 (Burchmore et al., 2003).

Expression of NT9/AT-D and NT10/AT-B during the Trypanosome Life Cycle. To assess expression of *NT9/AT-D* and *NT10/AT-B* in the various *T. brucei* life cycle stages, RT-PCR was performed on mRNA isolated from cultured procyclic forms (PC, s927), cultured long-slender forms (LS, EATRO 427 monomorphic strain) and short-stumpy bloodstream forms (SS, s927 pleomorphic strain) isolated from infected mice at peak parasitemia. The nucleobase transporter gene *TbNBT1* served as positive control because it is known to be expressed in PC and LS forms (Burchmore et al., 2003). PCR products of the predicted size for *NT9/AT-D*, *NT10/AT-B*, and *TbNBT1* were identified from mRNA

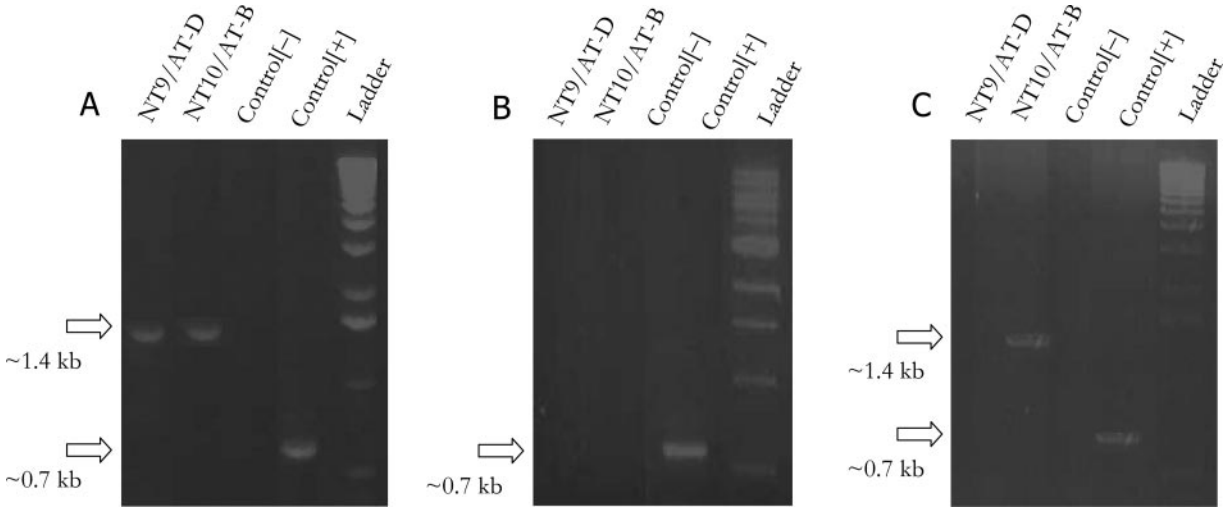

Fig. 1. Expression of *NT10/AT-B* and *NT9/AT-D* in different life-cycle stages of *Trypanosoma brucei brucei*. A, amplification of the NT9/AT-D and *NT10/AT-B* fragments from procyclic forms of *T. brucei brucei* s427 with the expected size of ~1.4 kb. B, represents the gel using long-slender bloodstream forms, showing no bands for *AT-B* or *AT-D*. C, amplification of *NT10/AT-B* fragment from short-stumpy bloodstream form of *T. brucei* 927 with the expected size of ~1.4 kb. The same fragment of *TbNBT1* (positive control) was amplified in all stages of the life cycle with a size of ~0.7 kb. The Invitrogen 1-kb DNA ladder was used to estimate sizes. Negative control was a parallel preparation produced in the absence of reverse transcriptase.

of PC trypanosomes (Fig. 1A), whereas only the *TbNBT1* product was amplified from LS mRNA (Fig. 1B). The presence of a high proportion of SS in the infected blood preparation was confirmed using the NAD diaphorase assay (Vickerman, 1985). All procyclic *T. brucei brucei* were stained in this assay, and more than 80% of parasites in the SS preparation (see Fig. 2 of Supplementary material). Because the LS trypanosomes do not express *NT9* or *NT10*, any product amplified with *NT9* or *NT10* primers must be derived from SS. Only *NT10/AT-B* and *TbNBT1* expression was detected in this preparation (Fig. 1C).

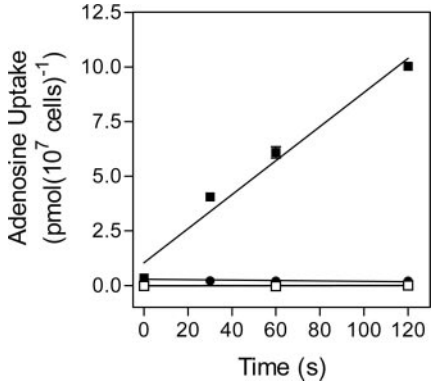

Fig. 2. Transport of [^{3}H]adenosine by AT-B in *S. cerevisiae* strain MG887-1. Uptake of 0.25 μM [^{3}H]adenosine was linear over 120 s (■: r^2 = 0.96, linear regression). In the presence of 1 mM unlabeled adenosine (□) or the control (pDR195 vector, ●), uptake was not significantly different from zero ($P > 0.4$ and $P > 0.2$, respectively; F test).

Characterization of NT10/AT-B by Heterologous Expression in *S. cerevisiae* and the Corresponding Transport Activity in *T. b. brucei*. Expression of *NT10/AT-B* in *S. cerevisiae* strain MG887-1 enabled these cells to rapidly accumulate 0.25 μM [^{3}H]adenosine (Fig. 2), with a rate of 0.080 ± 0.011 pmol/10^7 cells/s, and linear for at least 120 s. This accumulation, which was absent in cells transformed with the same vector without the *NT10/AT-B* insert, seemed to be mediated by a high-affinity transporter, in that it was completely inhibited by coincubation with 1 mM unlabeled adenosine (Fig. 2). The K_m (adenosine) for this transporter was determined as 0.41 ± 0.03 μM (n = 5), using a very low concentration of 0.015 μM [^{3}H]adenosine supplemented with increasing concentrations of unlabeled adenosine (Fig. 3A). K_m values were further determined for [^{3}H]inosine, [^{3}H]tubercidin, and [^{3}H]3-deazaadenosine (Fig. 3, B–D; Table 1). For each of these permeants, K_i values for adenosine were also determined as a positive control, yielding values close to the [^{3}H]adenosine K_m value. The K_i value for guanosine was 2.4 ± 0.28 μM (n = 3).

The results show that although NT10/AT-B has a high affinity for purine nucleosides, this is much reduced when the H-bond-accepting ring nitrogen residues, N3 and N7, are replaced by carbon atoms. We further determined that the high-affinity binding of TbNT10/AT-B is to a large extent dependent on a strong interaction with the 3′-hydroxyl group [K_i (3′-deoxyadenosine) = 31 ± 6.0 μM; n = 4], whereas the 2′-deoxy and 5′-deoxy groups were much less important for binding [K_i (2′-deoxyadenosine) = 1.0 ± 0.3 μM (n = 4); K_i (5′-deoxyadenosine) = 0.9 ± 0.1 μM (n = 3)]. Pyrimidine

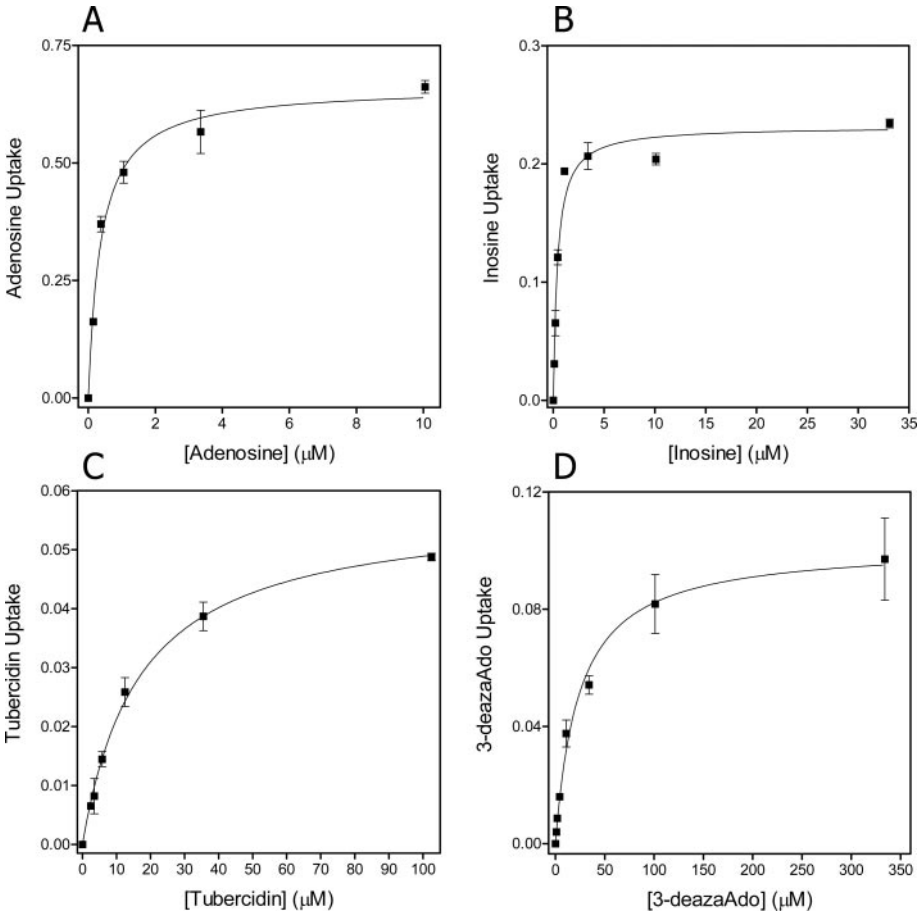

Fig. 3. Transport of 0.05 μM [^{3}H]adenosine (A), 0.1 μM [^{3}H]inosine (B), 2.0 μM [^{3}H]tubercidin (C), and 1.0 μM [^{3}H]3-deazaadenosine by *S. cerevisiae* transformed with pDR195:NT10 (D). Representative experiments are shown, each performed in triplicate, out of a total of three to four independent K_m determinations. Label concentrations were chosen well below anticipated K_m values. Transport was expressed as picomoles per 10^7 cells per second.

nucleosides and xanthosine had no effect on NT10/AT-B-mediated transport, and affinity for adenine and hypoxanthine was very low, with mean K_i values of 150 ± 42 and 320 ± 80 μM, respectively ($n = 3$).

Procyclic Trypanosomes Express Multiple Adenosine Transport Activities. Because *NT10/AT-B* is expressed in procyclic trypanosomes and pharmacologically distinct from the P1 activity of LS *T. brucei brucei* (Table 1), the question arises whether PC P1 is encoded by NT10/AT-B. A reinvestigation of adenosine transport in PC *T. brucei brucei* found that PC P1 is highly sensitive to 5′-deoxyadenosine (Fig. 4A) and displayed a K_m (adenosine) value of 0.15 ± 0.02 μM ($n = 4$), significantly lower than for NT10/AT-B ($P < 0.001$, Student t test). The observed K_m value could be the net result of adenosine uptake through multiple transporters with different K_m values. In four independent experiments, dipyridamole inhibited adenosine in procyclic *T. brucei brucei* by ~50% (Fig. 4A), with a K_i value of 0.64 ± 0.09 μM. Transport of 0.015 μM [^{3}H]adenosine was inhibited by unlabeled adenosine and 2′-deoxyadenosine in a monophasic way, with a K_i value for 2′-deoxyadenosine of 0.42 ± 0.04 μM (Fig. 4B).

***NT9/AT-D* Encodes a Purine Nucleoside Transporter with Exceptionally High Affinity for Adenosine.** No transport of [^{3}H]uracil, [^{3}H]hypoxanthine, or [^{3}H]adenine could be detected in yeast MG887-1 transformed with pDR195:AT-D (time course over 5 min; label concentration, 0.5 μM). Likewise, no transport of 2.5 μM [^{3}H]pentamidine was observed, even over 180 min (data not shown). However, transport of 0.25 μM [^{3}H]adenosine was shown to be linear over 120 s, with a rate of 0.0079 ± 0.0005 pmol/10^7 cells/s (linear regression, $r^2 > 0.99$), which was >95% inhibited by 1 mM unlabeled adenosine. As it was becoming clear that AT-D encodes an extremely high-affinity transporter, the experiment was repeated with 0.015 μM [^{3}H]adenosine, a concentration required to obtain valid K_m and K_i values for this transporter. Figure 5A shows that transport was linear for at least 4 min ($r^2 = 0.97$), and although it was only 65% inhibited by 1 mM adenine, it was fully inhibited by 1 mM unlabeled adenosine. Likewise, transport of 0.25 μM [^{3}H]inosine by *AT-D*-transformed yeast was fully saturable (Fig. 5B), with a rate of 0.0096 ± 0.0005 pmol/10^7 cells/s.

NT9/AT-D displayed extremely high affinity for adenosine, approximately 40-fold higher than for inosine, but the V_{max} was much higher for inosine (Fig. 6, Table 1). The transporter displayed very low affinity to nucleobases, including adenine (Table 2).

Substrate Binding by NT9/AT-D. The model for substrate binding by P1-type transporters (de Koning and

TABLE 1

K_m and V_{max} values for P1-type transporters

P1 activity, traditionally ascribed to the TbNT2 gene product and related genes on chromosome 2, and possibly made up of the combined flux through several different P1-type transporters (Sanchez et al., 2002), was measured in *T. brucei brucei* bloodstream forms as described previously (de Koning and Jarvis, 1999). *NT9/AT-D* and *NT10/AT-B* were expressed in *S. cerevisiae*, and transport of the various permeants measured as described under *Materials and Methods*. Results are the average and S.E. of three to five experiments, each performed in triplicate.

	P1 (LS)	P1 (PC)	NT10/AT-B	NT9/AT-D
Adenosine				
K_m (μM)	0.38 ± 0.10[a]	0.26 ± 0.02[b]	0.41 ± 0.03	0.068 ± 0.013
V_{max} (pmol/10^7 cells/s)	2.8 ± 0.4	0.63 ± 0.18	0.53 ± 0.08	0.013 ± 0.006
V_{max}/K_m	7.4	2.4	1.28	0.19
Inosine				
K_m (μM)		0.36 ± 0.04[b]	0.53 ± 0.06	2.75 ± 0.71
V_{max} (pmol/10^7 cells/s)		0.40 ± 0.02	0.25 ± 0.05	0.12 ± 0.04
V_{max}/K_m		1.1	0.48	0.044
Tubercidin				
K_m (μM)	5.5 ± 1.0		15 ± 1.5	
V_{max} (pmol/10^7 cells/s)	0.43 ± 0.10		0.25 ± 0.06	
V_{max}/K_m	0.077		0.016	
3-Deazaadenosine				
K_m (μM)	83 ± 17[c]		26 ± 4.0	
V_{max} (pmol/10^7 cells/s)			0.16 ± 0.04	
V_{max}/K_m			0.006	

[a] From de Koning and Jarvis (1999).
[b] From de Koning et al. (1998).
[c] K_i value from de Koning and Jarvis (1998)

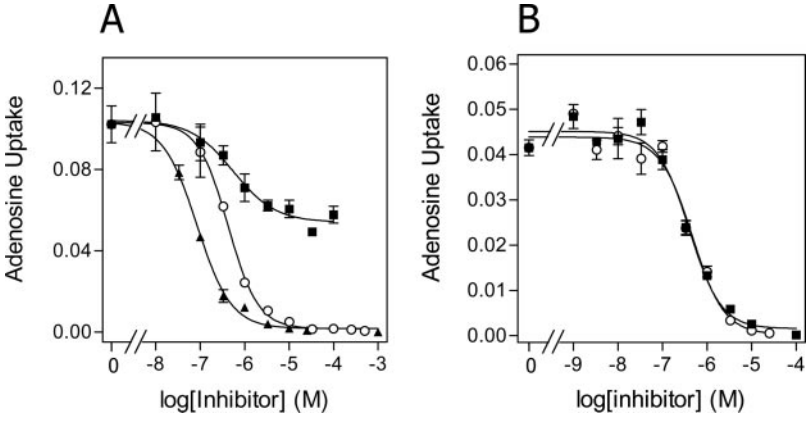

Fig. 4. Inhibition of [^{3}H]adenosine transport by dipyridamole and 5′-deoxyadenosine in procyclic and long-slender bloodstream forms. A, uptake of 0.05 μM [^{3}H]adenosine over 10 s in procyclic *T. brucei brucei* s427 in the presence or absence of various concentrations of dipyridamole (■), 5′-deoxyadenosine (○) and adenosine (▲). B, identical experiment using LS bloodstream forms from *T. brucei brucei* s427. Uptake was expressed as picomoles per 10^7 cells per second.

Jarvis, 1999; de Koning, 2001) predicts a similar binding energy for inosine and adenosine. We therefore undertook to investigate the molecular basis of the uniquely high adenosine binding energy (ΔG^0) of 42.5 kJ/mol. Whereas the ribose group of adenosine is known not to be involved in binding of the P2 transporter (de Koning and Jarvis, 1999), the ribose moiety made an important contribution to binding NT9/AT-D. 3′-Deoxyadenosine (cordycepin) and 5′-deoxyadenosine both displayed significantly reduced affinity for this transporter compared with adenosine ($P < 0.02$; Student t test) (Fig. 7A), corresponding with a reduced Gibbs free energy $\delta(\Delta G^0)$ of 11.5 and 4.0 kJ/mol, respectively. In contrast, the 2′-deoxy group was not required for binding, with the K_i value for 2′-deoxyadenosine not significantly different from the K_m value for adenosine (Table 2; Fig. 7A).

Of the functional groups of the purine ring itself, N3 and N7 contributed significantly to binding ($P < 0.05$), with $\delta(\Delta G^0)$ values of 3.95 and 9.8 kJ/mol, respectively, relative to adenosine, calculated from the K_i values of 3-deazaadenosine and tubercidin (7-deazaadenosine)(Fig. 7B). Any contribution of N1 to adenosine binding was found to be small: 1-deazaadenosine displayed a K_i value of 0.21 ± 0.07 μM, which was not statistically different from the adenosine K_m. The calculated $\delta(\Delta G^0)$ for 1-deazaadenosine was 2.9 kJ/mol, possibly attributable to a weakening of the N3 H-bond acceptor in 1-deazaadenosine rather than disruption of a weak H-bond at N1 itself. In contrast, the 6-position amine group seemed to make a major contribution, with nebularine (purine riboside) and 6-chloropurine riboside displaying significantly higher K_i values ($P < 0.02$ and $P < 0.01$, respectively) than the adenosine K_m (Fig. 7B), resulting in $\delta(\Delta G^0)$ values of 10.5 and 13.5 kJ/mol. It is possible that the partial negative charge of the chloride residue in 6-chloroadenosine has an energetically negative effect on the binding energy, in that the 6-amine group presumably functions as a H-bond donor to a partially negative charge in the binding pocket. Figure 8 summarizes the model for adenosine binding by NT9/AT-D, using the mean $\delta(\Delta G^0)$ value of nebularine and 6-chloroadenosine for the interaction energy at position 6. According to this model, the sum of the Gibbs free energy for the individual interactions is 41.3 kJ/mol, which is within 3% of the ΔG^0 calculated from the adenosine K_m (Table 2).

It could further be noted from the results listed in Table 2 that substitutions at position 2 of the purine ring are generally unfavorable. Guanosine displayed a significantly lower K_i value than inosine K_m ($P < 0.05$), and the addition of a nitro group at position 2 reduced the binding energy of adenosine by more than 18 kJ/mol.

Discussion

The purine salvage system, prominently including the primary step, transmembrane transport, has received much

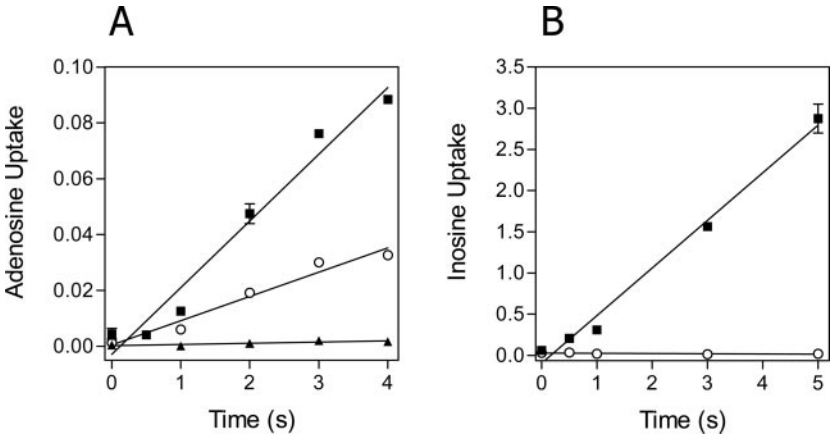

Fig. 5. Transport of purine nucleosides by NT9/AT-D expressed in *Saccharomyces cerevisiae.* A, transport of 0.015 μM [^{3}H]adenosine in the presence of 1 mM adenine (○), 1 mM adenosine (▲), or without inhibitor (■). B, transport of 0.25 μM [^{3}H]inosine in the presence (○) or absence (■) of 1 mM unlabeled adenine. Both experiments were performed in triplicate. Error bars are S.E. and, when not shown, fall within the symbols. Transport in the presence of excess permeant was not significantly different from zero ($P > 0.05$; F test). Transport units are picomoles per 10^7 cells.

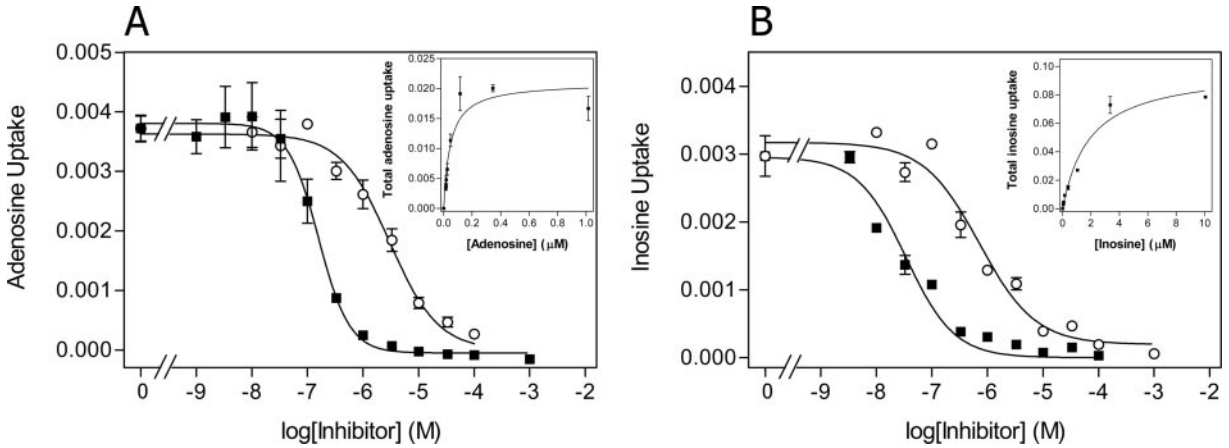

Fig. 6. Determination of adenosine and inosine K_m values for NT9/AT-D expressed in yeast. A, uptake of 0.015 μM [^{3}H]adenosine in the presence of various concentrations adenosine (■) or inosine (○). *Inset*, conversion of the adenosine inhibition data to a Michaelis-Menten curve. B, uptake of 0.05 μM [^{3}H]inosine in the presence of various concentrations of adenosine (■) or inosine (○). Inset, conversion of the inosine inhibition data to a Michaelis-Menten curve. Both experiments were performed in triplicate; error bars are S.E. Transport units are picomoles per 10^7 cells per second.

attention in many pathogenic organisms, both as drug target and as a conduit for the delivery of novel therapeutic nucleoside analogs (reviewed by de Koning et al., 2005; Lüscher et al., 2007). Similar reasoning has created much interest in the nucleoside transporters in neoplastic tissues (Huang et al., 2004) and in viral infections (Pastor-Anglada et al., 2005). The *Trypanosoma brucei* nucleoside transporters have thus far been divided in P1- and P2-type transporters, according to the classification introduced by Carter and Fairlamb (1993). Both P1 and P2 transporters efficiently transport numerous purine nucleoside analogs, whereas P2 additionally transports the first-line sleeping sickness drugs pentamidine and melarsoprol, which make the *T. brucei* nucleoside transporters of great pharmacological significance (Geiser et al., 2005; Lüscher et al., 2006). The work on *T. brucei brucei* purine transporters has contributed very significantly to our understanding of the ENT family (de Koning et al., 2005). We now report the cloning and characterization of an ENT nucleoside transporter with a uniquely high affinity for adenosine and the elucidation of the mechanism of binding responsible for this phenomenon.

Biochemical Basis of Unique Substrate-Binding Profile by NT9/AT-D. We found that NT9/AT-D, when expressed in *S. cerevisiae*, displayed an extraordinarily high affinity for adenosine and 2′-deoxyadenosine. We are not aware of any other nucleoside transporter with as high an affinity for its substrate(s). It also displays 40-fold lower affinity for inosine, which sets it apart in the P1-class of ENT transporters, although phylogenetic analysis clearly placed it in this category (de Koning et al., 2005). The strong selectivity of adenosine over inosine, in particular, could not be explained on the basis of the binding model for P1 transporters based on kinetic analysis of the P1-activity in LS trypanosomes (de Koning and Jarvis, 1999; de Koning, 2001). We thus examined which functional groups contribute to adenosine and inosine binding to NT9/AT-D. We found that in addition to the established four interactions between P1 transporters and purine nucleosides, 3′-hydroxy, 5′-hydroxy, N3 and N7—which do not differentiate between oxypurine and aminopurine nucleosides—NT9/AT-D also interacts strongly with the 6-position amine group of adenosine, presumably through one or more hydrogen bonds. This interaction explains the much higher affinity for adenosine than other P1 transporters, which apparently lack this interaction. In addition, a possible interaction with the pyrimidine N1 of adenosine was identified, though this did not reach statistical significance. The binding of the N1-C6-NH_2 motif is the characteristic signature of P2 that enables it to bind diamidines and melaminophenyl arsenicals as well as aminopurines (de Koning and Jarvis, 1999; Barrett and Fairlamb, 1999).

TABLE 2

Kinetic parameters and Gibbs free energies of the interactions of potential substrates and inhibitors with NT9/AT-D

Compound	K_i	n	ΔG^0
	μM		*kJ/mol*
2′-Deoxyadenosine	0.046 ± 0.006	3	43.5
Adenosine	0.068 ± 0.013[a]	4	42.5
1-Deazaadenosine	0.21 ± 0.07	4	39.7
3-Deazaadenosine	0.31 ± 0.09	3	38.6
5′-Deoxyadenosine	0.32 ± 0.07	3	38.5
Adenine arabinoside (AraA)	1.4 ± 0.3	3	34.8
Tubercidin	2.3 ± 0.2	4	33.4
Inosine	2.8 ± 0.7[a]	3	33.0
Nebularine	4.0 ± 1.0	3	32.0
7-Deaza-2′-deoxyadenosine	4.0 ± 0.6	3	32.0
Cordycepin	5.9 ± 1.4	3	31.0
Guanosine	6.2 ± 0.6	3	30.9
6-Mercaptopurine riboside	6.6 ± 2.5	2	30.7
6-Chloropurine riboside	12.8 ± 0.2	3	29.0
2-Nitropurine riboside	80 ± 12	2	24.1
2-Nitroadenosine	88 ± 10	2	24.3
Adenine	148 ± 32	3	22.7
Uridine	235 ± 39	3	21.5
Hypoxanthine	320 ± 80	3	20.7
Thymidine	510 ± 35	3	19.6
Xanthosine	>1000	2	

[a] K_m.

Assuming an identical orientation for oxypurines and aminopurines in the NT9/AT-D binding pocket, inosine interactions would involve the 3′-hydroxy, 5′-hydroxy, N3, and N7 functional groups with energies similar to those for adenosine. The 6-keto group does not seem to be involved in binding, judging from the near-identical binding energies for 6-mercaptopurine riboside and inosine (Table 2). It is also not likely that the lactam hydrogen N1(H) engages in hydrogen bonding, because nebularine and inosine have almost the same calculated binding energy (Table 2). The difference between the adenosine and inosine binding energies, 9.6 kJ/mol, can thus be explained by the existence of a strong H-bond between the 6-amino group and the NT9/AT-D binding pocket, and a possible weak H-bond with N1. This effectively constitutes a fusion of the P1 and P2 binding motifs.

Demonstration of Multiple Distinct P1-type Transport Activities in *T. brucei*. A P1-type adenosine and inosine transport activity was first described in *T. brucei brucei*

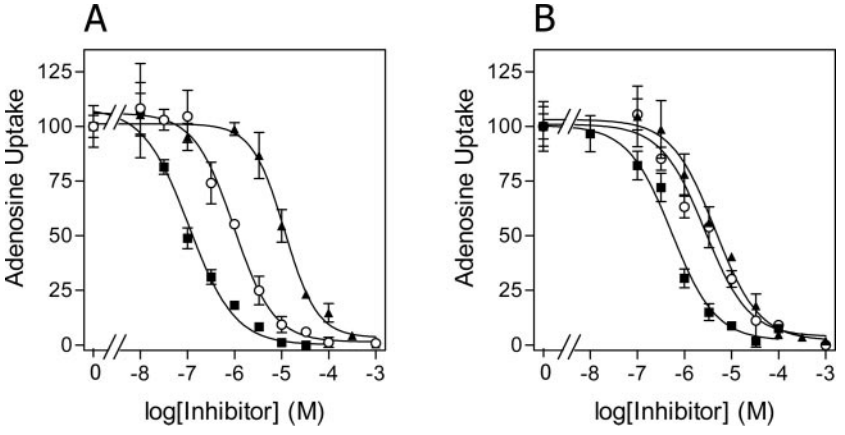

Fig. 7. Inhibition of adenosine transport by NT9/AT-D expressed in *S. cerevisiae*. Uptake of 0.05 μM [^{3}H]adenosine in yeast strain MG887-1 transformed with pDR195:AT-D was determined over 60 s in the presence of various purine analogs. Transport was expressed as percentage of control value, being transport in the absence of inhibitor. A, 2′-deoxyadenosine (■), 3′-deoxyadenosine (▲), 5′-deoxyadenosine (○). B, 1-deazaadenosine (■), tubercidin (▲), nebularine (○). Data shown are combined from representative single experiments performed in triplicate.

bloodstream forms (Carter and Fairlamb, 1993) and later in procyclic trypanosomes (de Koning et al., 1998). To date, it has been unclear how many adenosine transporters are functionally expressed in the various life cycle forms, particularly because in trypanosomes, which express most genes constitutively in large polycistrons, the presence of mRNA does not necessarily indicate the presence of the protein product (Clayton, 2002). The number of distinct adenosine transporters is relevant from a pharmacological perspective, and certainly in the context of induction of drug resistance, but is difficult to assess on a protein level, although efforts to establish a *T. brucei brucei* plasma membrane subproteome for bloodstream forms are in progress (D. J. Bridges, A. R. Pitt, H. P. Voorheis, H. P. de Koning, and R. J. S. Burchmore, unpublished). Here, we show from both the inhibitor profiles of LS P1, PC P1, and NT10/AT-B expressed in yeast that 1) whereas NT10/AT-B encodes a P1-type transporter, it is not the P1 activity expressed in LS forms and 2) that the P1 transport activity in PC forms (de Koning et al., 1998) is subtly different from the corresponding transporter in LS forms.

This follows an earlier observation that substrate binding by LS P1, but not PC P1, is negatively affected by substitutions at position 2 of the purine ring (2-chloroadenosine; de Koning et al., 1998; de Koning and Jarvis, 1999) and that only PC P1 is sensitive to inhibition by the nucleoside transport inhibitor dipyridamole (de Koning et al., 1998). We now show that adenosine transport in procyclics is inhibited by 5′-deoxyadenosine in a monophasic manner, with a K_i value consistent with this transporter being NT10/AT-B (but very different from the 5′-dAdo K_i value of LS P1) but is only partially inhibited by dipyridamole. These observations strongly suggest that procyclic trypanosomes express at least two highly similar purine nucleoside transporters, including *NT10*, distinguished by their sensitivity to dipyridamole and different from the P1 adenosine transporter of *T. brucei brucei* LS bloodstream forms.

These conclusions are consistent with the results of the RT-PCR analysis, which indicate that *NT10/AT-B* is not expressed in LS forms, but that NT10 mRNA is present in procyclic and short stumpy forms, and in agreement with the observations of Sanchez et al. (2004). In unpublished work, Roditi and coworkers constructed an NT10−/− *T. brucei brucei* line, which was only partly resistant to the nucleoside antimetabolites tubercidin and cordycepin (I. Roditi, personal communication). This observation is further evidence that transport-related resistance against purine antimetabolites will be very hard to induce in *Trypanosoma brucei* spp. after unsuccessful attempts to induce allopurinol resistance in bloodstream form *T. brucei brucei* (Natto et al., 2005).

NT9/AT-D mRNA was also found in procyclic trypanosomes, but we were unable to demonstrate the activity of this transporter in PC *T. brucei brucei*, even when using very low concentrations of labeled adenosine to favor uptake through this carrier. This may be because the expression of *NT9/AT-D* is relatively low under standard culture conditions, coupled with the low capacity associated with such high

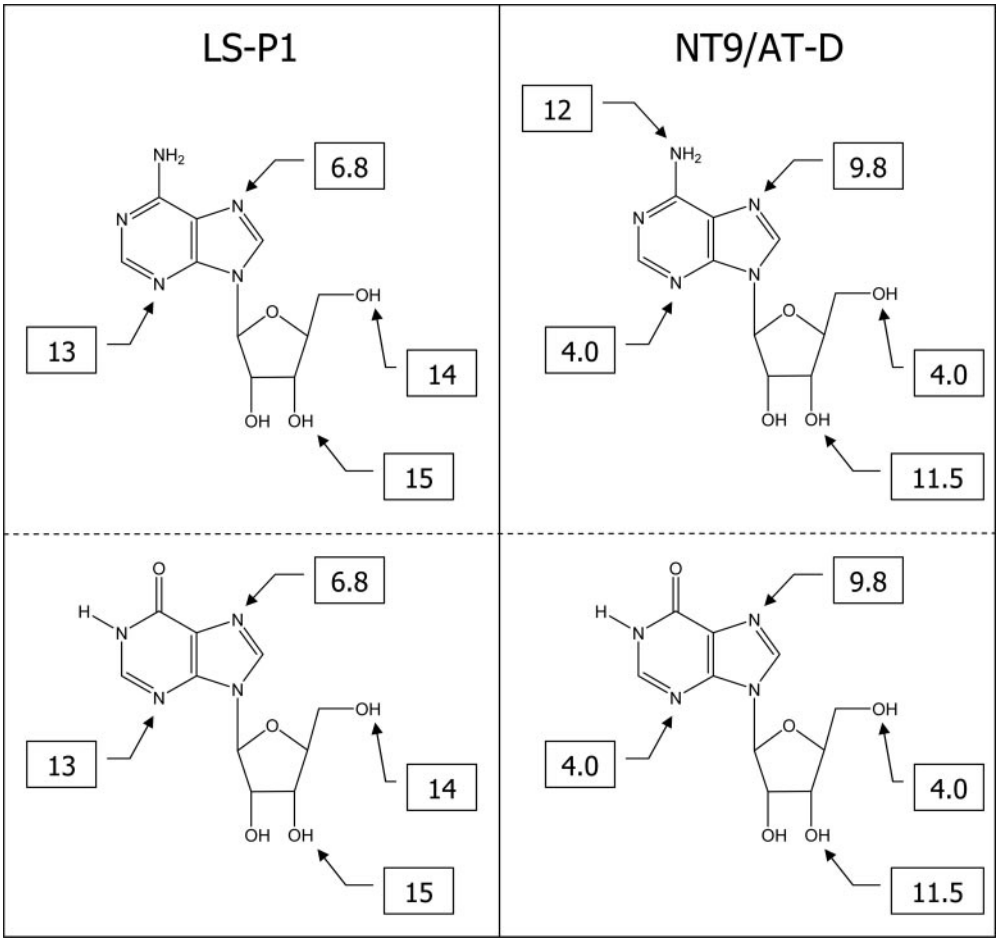

Fig. 8. Model for the binding of adenosine (top) and inosine (bottom) by the P1 transport activity measured in long-slender bloodstream forms or by NT9/AT-D expressed in *S. cerevisiae* MG887-1. Estimated Gibbs free energy of interaction, ΔG^0, for the indicated functional groups is given in kilojoules per mole.

substrate affinity. However, it is likely that *NT9/AT-D* is of physiological value to the procyclic parasite, which, in its environment of the tsetse fly midgut, must periodically experience very low purine concentrations. We have previously demonstrated that a high-affinity hypoxanthine transporter of procyclic *T. brucei brucei* is up-regulated as a result of purine-depleted conditions (de Koning et al., 2000). Similar up-regulation of purine salvage proteins has been demonstrated in several other kinetoplastids (Gero et al., 1997; Seyfang and Landfear, 1999; Liu et al., 2005).

We were unable to definitively identify the second P1-type transport activity observed in procyclic cells. Phylogenetic analysis of ENT family genes in *T. brucei brucei* suggests the existence of eight such genes (de Koning et al., 2005): *NT9/AT-D*, *NT10/AT-B*, and the cluster of six highly similar copies, *NT2* to *NT7*, on chromosome 2, of which NT3 and NT4 are reportedly nonfunctional and NT5 to NT7 are mostly expressed in bloodstream forms rather than procyclic forms (Sanchez et al., 2002). By a process of elimination, we thus arrive at the hypothesis that NT2 and NT10/AT-B together constitute adenosine transport in procyclic *T. brucei brucei* under purine replete conditions.

In conclusion, we have cloned and characterized two nucleoside transporters from African trypanosomes and shown the biochemical basis of their unique substrate binding profiles. We have further shown that these transporters mediate the uptake of pharmacologically relevant antimetabolites and provided new insights in the complexities of nucleoside transporter usage by this protozoan parasite.

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