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Adenosine Transporters in Bloodstream Forms of *Trypanosoma brucei brucei*: Substrate Recognition Motifs and Affinity for Trypanocidal Drugs

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ABSTRACT

Adenosine influx by *Trypanosoma brucei brucei* P1 and P2 transporters was kinetically characterized. The P1 transporter displayed a higher affinity and capacity for adenosine (K_m = 0.38 ± 0.10 μM, V_{max} = 2.8 ± 0.4 pmol · 10^7 cells^{-1} · s^{-1}) than the P2 transporter (K_m = 0.92 ± 0.06 μM, V_{max} = 1.12 ± 0.08 4 pmol · 10^7 cells^{-1} · s^{-1}). To formulate a structure-activity relationship for the interaction of adenosine with the transporters, a series of analogs were evaluated as potential inhibitors of adenosine transport, and the K_i values were converted to binding energy. The P1 transporter was found to be selective inhibited by purine nucleosides (K_i ~ 1 μM for inosine and guanosine), but nucleobases and pyrimidines had little effect on P1-mediated transport. The P1 transporter appears to form hydrogen bonds with N3 and N7 of the purine ring as well as with the 3′ and 5′ hydroxyl groups of the ribose moiety, with apparent bond energies of 12.8 to 15.8 kJ/mol. The P2 transporter, in contrast, had high-affinity (K_i = 0.2–4 μM) for 6-aminopurines, including adenine, 2′-deoxyadenosine, and tubercidin, but not for any oxopurines. The main interaction of adenosine with the P2 transporter is suggested to be via hydrogen bonds to N1 and the 6-amino group. Additional π-π interactions of the purine ring and electrostatic interactions with N9 may also be important. The predicted substrate recognition motif of P2, but not of P1, corresponds to parts of the melaminophenylarsenical and diamidine molecules, confirming the potent inhibition observed with these trypanocides for P2-mediated adenosine transport (K_i = 0.4–2.4 μM).

African trypanosomes are the causative agents of sleeping sickness, and the corresponding diseases in livestock and game animals continue to be a major public health and veterinary problem in many parts of Africa (Wery, 1991; Kuzoe, 1993). These protozoan live freely in the bloodstream and (in late-stage infections) the cerebrospinal fluid of the host. Treatment of infections with African trypanosomes in humans is limited to chemotherapy with diamidines (pentamidine), suramin, melaminophenylarsenicals (melarsoprol), and *dl*-α-difluoromethylornithine (DFMO), and only the latter two are effective against late-stage sleeping sickness (Pepin and Milford, 1994), with melarsoprol being the drug of choice against the acute form of the disease caused by *Trypanosoma brucei rhodesiense* (Bacchi et al., 1990). Because existing trypanocides cause considerable side effects and because resistance to them is a serious problem (Kayembe and Wéry, 1972; Dukes, 1984; Bacchi et al., 1990, 1993; Bacchi, 1993), there is an urgent need for new chemotherapeutic approaches.

During the past few years, interest in the purine nucleoside transporters of trypanosomes has intensified, for the following reasons. First, trypanosomes, like all protozoan parasites, are auxotrophic for purines and rely entirely on salvage from the host environment for their purine supply (Hammond and Gutteridge, 1984; Hassan and Coombs, 1988). As the first step in purine salvage is translocation of the purine across the cell membrane, the transport routes are potential targets for chemotherapy. *T. b. brucei* bloodstream forms express at least two different nucleoside transporters (Carter and Fairlamb, 1993), designated P1 and P2, as well as two nucleobase transporters (De Koning and Jarvis, 1997b, 1998). However, we have only limited information on the structure-activity relationships of the different transporters. Somoza et al. (1998) recently demonstrated that block of purine salvage in protozoa through rational design of novel drugs is practicable. A second reason for intense interest in *T. b. brucei* purine nucleoside transporters is that they have been implicated in the uptake of trypanocidal drugs, and changes to these carriers can lead to drug resistance (Carter and Fairlamb, 1993; Barrett et al., 1995; Carter et al., 1995; Ross and Barns, 1996). Purine transporters have also been suggested to be an ideal conduit for the internalization of new designer drugs (Tye et al., 1998).

To exploit the trypanosome purine transporter for the delivery of new drugs (e.g., cytotoxic nucleoside analogs), several conditions must be met to confer selectivity and efficacy;

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these include 1) high affinity of the trypanocide for the parasite transporter, combined with 2) low affinity for the mammalian transporters, 3) low abundance of competing substrates for the trypanosome transporters in its natural environment, and, ideally, 4) concentrative rather than equilibrative uptake. Trypanosome purine transporters appear to satisfy at least the last two requirements. Purine concentrations in the blood are low (up to 1 μM; Plagemann et al., 1988), and *T. b. brucei* nucleoside and nucleobase transporters are protonmotive force driven (De Koning and Jarvis, 1997a,b, 1998; De Koning et al., 1998).

To investigate whether drug delivery through the *T. b. brucei* purine nucleoside transporters could meet the first two criteria as well, we determined the kinetic constants for transport and evaluated a series of nucleosides and nucleobases as potential inhibitors of the P1 and P2 transporters. Based on the inhibition data, a structure-activity relationship for the binding of analogs to the transporters was formulated to identify and compare the specific substrate recognition motifs for the P1 and P2 transporters. In addition, the affinities of existing arsenical and diamidine trypanocides for the different transporters were determined, as well as for potentially chemotherapeutic nucleoside analogs such as tubercidin (7-deazaadenosine), ganciclovir, ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide), and formycins A (3-[β-D-ribofuranosyl]-pyrazolo[4,3-*d*]7-amine-pyrimidine) and B (3-[β-D-ribofuranosyl]pyrazolo[4,3-*d*]6*H*-7-pyrimidone).

Materials and Methods

Trypanosomes. *T. b. brucei* (strain 427) from frozen stocks were grown in CD rats (Charles River), and blood was collected through exsanguination. Trypanosomes were separated from blood cells on a DE52 (Whatman) anion exchange column (Lanham, 1968), counted in a hemocytometer, washed twice with the assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM NaH_2PO_4, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$, and 14 mM glucose, pH 7.3), and resuspended at 10^8 cells/ml. The trypanosomes were kept at 25°C at all times. These conditions are essential because previous studies have demonstrated that the membrane depolarizes when the trypanosomes are prepared and stored at 4°C (Defrise-Quertain et al., 1996). At the end of each experiment, cell viability and motility were checked under a phase-contrast microscope.

Transport Assays. The uptake of adenosine and hypoxanthine was assessed as previously described (De Koning and Jarvis, 1997a,b; De Koning et al., 1998). Briefly, 100 μl of bloodstream forms of *T. b. brucei* in assay buffer (10^7 cells) was mixed with 100 μl of assay buffer containing [2,8,5′-^{3}H]adenosine (2012 GBq/mmol; NEN, Germany) or [8-^{3}H]hypoxanthine (999 GBq/mmol; Amersham Pharmacia Biotech, UK) and, where appropriate, test compound. The final concentrations of [^{3}H]adenosine and [^{3}H]hypoxanthine were 0.02 and 0.03 μM, respectively, unless otherwise indicated. These low concentrations of permeant were chosen to allow uptake to be determined at concentrations below the measured K_m value of the different transporters. Under these conditions, the initial rate of uptake will approximate the V_{max}/K_m ratio, and inhibitory effects of the test compounds are readily observed; the IC_{50} values will approach the K_i values (see eq. 1). If higher substrate concentrations were used, then higher concentrations of test compound are required to inhibit the uptake of radiolabeled substrate due to competition in binding between substrate and test compound. It may not always be possible to obtain the necessary high concentrations of the test compound due to insolubility; moreover, there is a risk of misclassifying inhibitory compounds as noninhibitors. After predetermined times, normally 10 s, uptake was stopped by the addition of ice-cold buffer containing 1 mM adenosine or 4 mM hypoxanthine, respectively, and centrifugation through an oil layer [7:1 (v/v) di-*n*-butyl phthalate/mineral oil]. The resulting pellet was dissolved in 0.5 M NaOH, mixed with scintillation fluid (Optiphase HiSafe III), and counted for radioactivity. To separately determine uptake by the P1 and P2 adenosine transporters, adenosine uptake was assessed in the presence of 100 μM adenine or inosine, respectively. Most test compounds, including diminazene aceturate (Berenil), were purchased from Sigma (Poole, UK). Pentamidine isothionate (Pentacarinat) was obtained from Rhone-Poulenc Rorer (Dagenham, UK). Ribavirin and dilazep were generous gifts from the Schering-Plough Research Institute (Kenilworth, NJ) and Hoffman-La Roche (Basel, Switzerland), respectively. Melarsoprol was obtained from May and Baker Ltd. (Dagenham, UK) as a 3.6% (w/v) solution in propylene glycol.

Data Analysis. All experiments were carried out in triplicate. Data were fitted to the appropriate equations with the use of the Enzfitter and Fig.P. software packages (Elsevier Biosoft, Cambridge, UK) to obtain K_m and IC_{50} values. IC_{50} values for compounds inhibiting the uptake of radiolabeled permeants were determined from full dose-response curves with a minimum of eight points spread over the relevant range. In all cases, the Hill coefficients were close to -1, consistent with competitive inhibition. Moreover, in previous studies, it has been demonstrated that a number of the compounds tested in the present study, including trypanocides, inhibited P1- or P2-mediated adenosine transport competitively (Carter and Fairlamb, 1993; Carter et al., 1995; De Koning et al., 1998). Thus, all the available evidence suggests that a simple model of competition with the binding site of the transporter is applicable and that the criteria for use of the Cheng and Prusoff equation to determine K_i value have been met. Hence, K_i values were calculated from the Cheng and Prusoff (1973) equation

$$K_i = IC_{50}/[1 + (L/K_m)] \quad (1)$$

in which L is the permeant concentration. It should be noted that the K_i value is an affinity constant and implies binding to the transporter but does not indicate that the ligand is being transported across the membrane. Free Gibbs energy ΔG^0 was calculated from

$$\Delta G^0 = -RT\ln(K_i) \quad (2)$$

in which R is the gas constant and T the absolute temperature. Errors given in tables and shown as bars in graphs are standard errors.

Results

Adenosine Transport in Bloodstream Forms of *T. b. brucei*. Total uptake of 0.2 μM [^{3}H]adenosine in bloodstream forms of *T. b. brucei* was determined using a rapid stop/oil spin protocol developed for procyclic forms of *T. b. brucei* (De Koning et al., 1998). Figure 1 demonstrates that adenosine transport was efficiently terminated by the addition of ice-cold 1 mM adenosine. Adenosine uptake was found to be linear for at least 50 s, with a rate of 0.47 ± 0.024 pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1} at 0.2 μM [^{3}H]adenosine (Fig. 1). The addition of 1 mM unlabeled adenosine completely inhibited the uptake of 0.2 μM [^{3}H]adenosine, indicating that the transport of adenosine occurs via a mediated pathway (Fig. 1).

Adenosine uptake was inhibited to different levels by adenine and inosine (Fig. 2). Adenine (100 μM) inhibited 0.02 μM [^{3}H]adenosine by 22.1 ± 3.0%, with a K_i value of 0.30 ± 0.02 μM ($n = 3$), whereas 100 μM inosine reduced uptake of [^{3}H]adenosine by 81.2 ± 2.2%, with a K_i value of 0.44 ± 0.10

μM ($n = 5$). In the presence of 100 μM inosine, the remaining adenosine flux was dose-dependently inhibited by adenine (Fig. 2). Conversely, inosine completely inhibited [^{3}H]adenosine uptake in the presence of 100 μM adenine (not shown). The Hill slopes for inhibition by either purine were consistently close to -1 (both -1.0 ± 0.1). These results confirm the conclusion of Carter and Fairlamb (1993) that bloodstream forms of *T. b. brucei* express two distinct adenosine transporters, designated P1 and P2, that are sensitive to inhibition by inosine and adenine, respectively. Figure 3 shows that the P1 transporter had both a higher affinity and a higher capacity for adenosine ($K_m = 0.35 \pm 0.14$ μM, V_{max} $= 2.2 \pm 0.2$ pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1}) than the P2 transporter ($K_m = 0.94 \pm 0.21$ μM, $V_{max} = 1.24 \pm 0.09$ 4 pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1}). The mean kinetic constants from three separate experiments were K_m of 0.38 ± 0.10 and 0.92 ± 0.06 μM and V_{max} of 2.8 ± 0.4 and 1.12 ± 0.08 pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1} for P1 and P2, respectively.

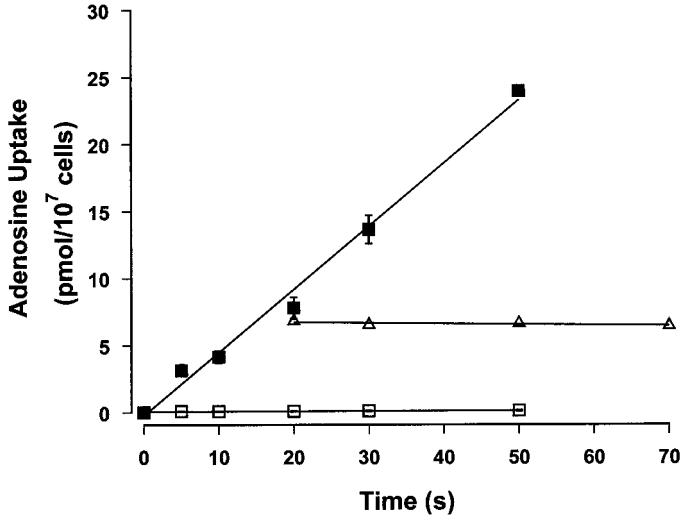

Fig. 1. Time course for [^{3}H]adenosine uptake by bloodstream forms of *T. b. brucei*. Uptake of [^{3}H]adenosine at a final concentration of 0.2 μM (■) was initiated by the addition of 10^7 cells to [^{3}H]adenosine layered over an oil mixture. At the indicated times, transport was stopped by the addition of 1 ml ice-cold stop solution (1 mM adenosine in assay buffer) and immediate centrifugation through the oil. Nonmediated influx of adenosine was assessed by determining the rate of 0.2 μM [^{3}H]adenosine uptake in the presence of 1 mM unlabeled adenosine (□). In an additional series (△), the cells were incubated with 0.2 μM [^{3}H]adenosine for 20 s, stopped with 1 ml of stop solution, and centrifuged at the indicated times (after 0, 10, 30, and 50 s). The rate of uptake after the addition of stop solution was not significantly different from zero.

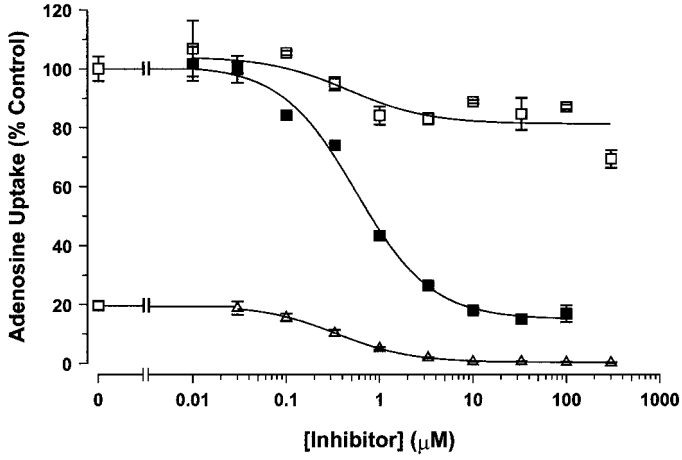

Fig. 2. Demonstration of two distinct adenosine transporters in bloodstream forms of *T. b. brucei*. Uptake of 0.02 μM [^{3}H]adenosine over 10 s was measured in the presence of increasing concentrations of inosine (■), adenine (□), or adenine in the presence of 100 μM inosine (△). IC_{50} values were 0.56 ± 0.07, 0.46 ± 0.25, and 0.34 ± 0.01 μM, respectively. Data points are the average of triplicate estimates from a single experiment.

Substrate Recognition by P1 and P2 Adenosine Transporters. P1- and P2-mediated transport of 20 nM [^{3}H]adenosine was assessed in the presence of 100 μM adenine or inosine, respectively. A range of potential substrates (naturally occurring purines and pyrimidines as well as purine analogs) was tested for their ability to inhibit P1 or P2 transport activity. Based on the inhibition data, a structure-activity relationship for the binding of nucleosides/nucleobases to the P1 and P2 adenosine transporters was formulated and a comparison was undertaken. The K_i values were determined from full dose-response curves and in triplicate (Fig. 4). Table 1 lists the K_i values along with their respective binding energies.

P1 Transporter. The P1 transport function was not inhibited by pyrimidine or purine nucleobases when tested at concentrations up to 1 mM. In contrast, adenosine influx via P1 was potently inhibited by a variety of purine nucleosides (Table 1), showing that the ribose moiety is essential for binding to P1. However, the 2′-hydroxyl group is not required because 2′-deoxyadenosine has, if anything, a slightly higher affinity than adenosine (0.19 ± 0.02 μM, Fig. 4A). Nevertheless, both 3′- and 5′-deoxyadenosine had a markedly reduced binding affinity for the P1 transporter ($K_i = 210 \pm 48$ and 100 ± 7 μM, respectively; Table 1). Clearly, both hydroxyl groups are involved in interactions with the transporter. For both ligands, the reduction of the binding energy ΔG^0 compared with adenosine of 15.1 and 14.1 kJ/mol for 3′- and 5′-deoxyadenosine, respectively, is consistent with the loss of one hydrogen bond between substrate and carrier. The involvement of the ribose group is confirmed by the inability of

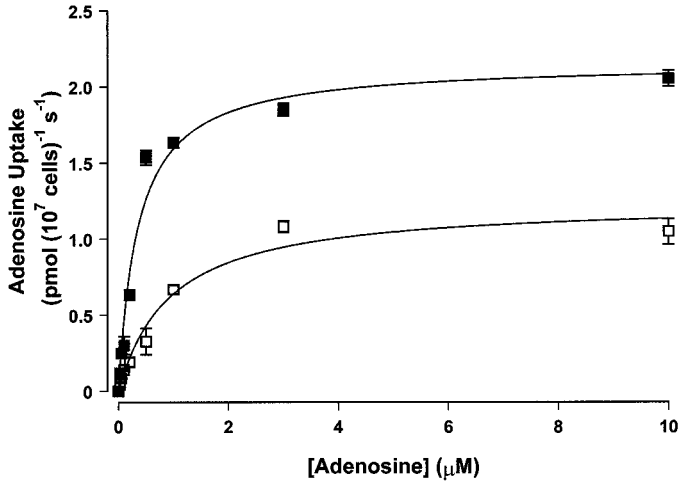

Fig. 3. Concentration dependence of adenosine influx by bloodstream forms of *T. b. brucei*. Initial rates of adenosine uptake were measured by incubating cells for 10 s with 0.02 μM [^{3}H]adenosine and 0 to 100 μM unlabeled adenosine in the presence of 100 μM adenine (■, P1 mediated) or 100 μM inosine (□, P2 mediated). The kinetic constants were determined by nonlinear regression using the Michaelis-Menten equation. Values for this experiment were $K_m = 0.35 \pm 0.14$ μM and $V_{max} = 2.2 \pm 0.2$ pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1} for P1 and 0.94 ± 0.21 μM and 1.24 ± 0.09 pmol $\cdot 10^7$ cells$^{-1} \cdot$ s^{-1} for P2. Data points are the average of triplicate determinations.

the acyclic guanosine analog ganciclovir to inhibit P1-mediated adenosine uptake. However, the strict selectivity for purines over pyrimidines (Table 1) showed that the aglycon part of the substrate was also involved in ligand/transporter interactions. This was further illustrated by the observation that ribose alone has no detectable effect on P1-mediated adenosine uptake, even at 1 mM (Table 1).

The group at position 6 of the purine ring did not form part of the binding recognition motif because guanosine (Fig. 4B) and inosine, each with a keto group at this position instead of the amine group of adenosine, were potent inhibitors of adenosine influx. However, the low affinity of 3-deazaadenosine (Fig. 4E) and ribavirin (structure; Fig. 5) demonstrates that the loss in nitrogen residue at position 3 is essential for high-affinity binding to the P1 transporter. The loss in binding energy of 13.5 and 10.8 kJ/mol, respectively, suggests the loss of one hydrogen bond between ligand and transporter. The somewhat higher binding energy for ribavirin than for 3-deazaadenosine might be explained if the nitrogen residue at position 4, unique to ribavirin, was able to form the same hydrogen bond as N3, albeit much weaker. The involvement of N3 in H-bonding is also apparent from the effect of substitutions on the adjacent position 2. Both a chloride (2-chloroadenosine, $K_i = 15 \pm 5.3$ μM) and, to a lesser extent, an amine (guanosine, $K_i = 1.8 \pm 0.3$ μM; Fig. 4B) would reduce the partial negative charge on N3 and weaken the hydrogen bond. The loss in ΔG^0 of 9.2 and 4.0 kJ/mol, respectively, does indicate a weakened rather than a disrupted hydrogen bond. Alternatively, the substitutions on position 2 could interfere with the H-bond to N3 through steric or electrostatic repulsion of the relevant amino acid residue of the transporter.

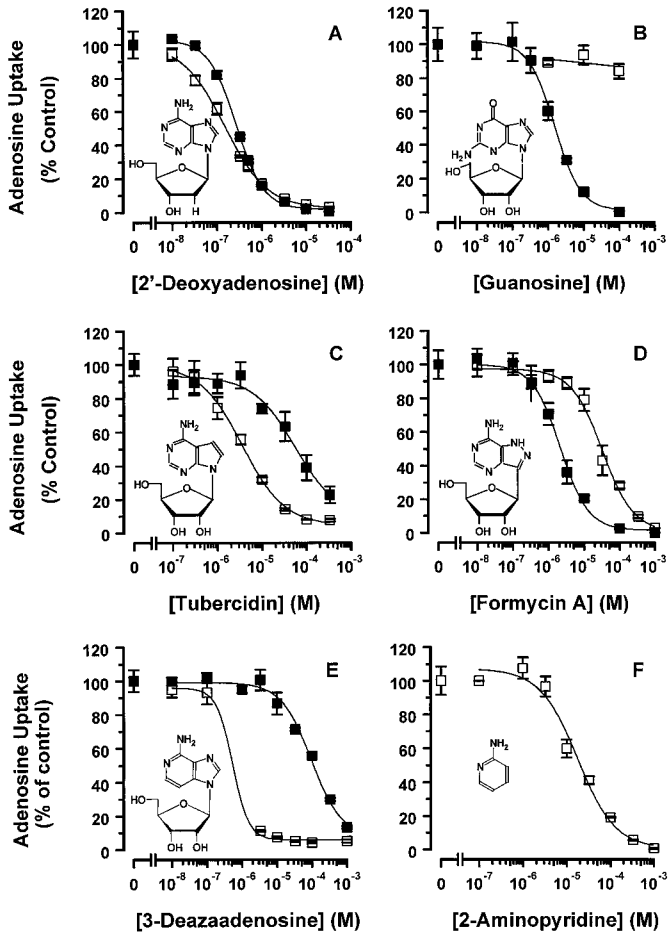

Fig. 4. Comparison of the effects of selected purine nucleosides on P1- and P2-mediated [^{3}H]adenosine transport. P1-mediated (■) and P2-mediated (□) adenosine flux were measured in the presence of 100 μM adenine or 100 μM inosine, respectively. Incubation time was 10 s, [^{3}H]adenosine concentration was 0.02 μM, and the data points are the average of triplicate determinations.

The low affinity of the P1 transporter for uridine (K_i = 830 ± 86 μM), and the corresponding reduction of 19.2 kJ in ΔG^0 compared with adenosine, is consistent with the loss of 2 H-bonds to the purine ring. This suggests that part of the imidazole ring is also involved in binding. The identification of which residues of the imidazole are essential for binding to the transporter was based on examination of the potency of formycins A and B (analogs of adenosine and inosine, respectively, with the N9 residue shifted to position 8, see Fig. 5), 8-azidoadenosine, and tubercidin (7-deazaadenosine) to inhibit P1-mediated transport. The affinity of the P1 transporter for tubercidin (Fig. 4C) was almost 200-fold less than that for adenosine, showing the importance of N7. The ΔG^0 for tubercidin (−23.4 kJ/mol) was similar to the values for 3-deazaadenosine, 3′deoxyadenosine, and 5′deoxyadenosine, consistent with each compound forming one H-bond less than adenosine with the P1 transporter. The P1 transporter displayed a relatively high affinity for formycin A (Fig. 4D) and formycin B (Table 1), indicating that neither C8 nor N9 is important for ligand binding. However, as a consequence of the substitution of an imidazole for a pyrazolo ring, the protonation state of N7 has largely shifted from unprotonated to protonated (Bzowska et al., 1992; Fig. 5). The energy required to shift the tautomeric equilibrium to N(2)H (equivalent to position 8 in adenosine, Fig. 5) may be responsible for the loss of ~3 kJ/mol in ΔG^0 of the formycins compared with adenosine (Table 1). The 25-fold decrease in potency of 8-azidoadenosine to inhibit adenosine uptake via P1 (compared with adenosine) is probably attributable to both its size and its resonance with the imidazole ring. This results in weakening the H-bond to N7 in a similar way as the substitutions on position 2 weaken the H-bond to N3.

P2 Transporter. In contrast to the P1 transporter, the P2 transporter does not require the presence of the ribose moiety for ligand binding. This is evident from the high-affinity interaction of adenine (see earlier), resulting in P2 being a mixed nucleoside/nucleobase transporter. It is therefore predicted that most substitutions at position 9 of the purine ring, or small alterations to the ribose group, will not significantly increase the K_i value compared with adenosine. The K_i value of 0.23 ± 0.04 μM for 2′-deoxyadenosine is consistent with this prediction (Fig. 4A). Indeed, the presence of a 2′ hydroxyl group seems to reduce the binding energy by 3.4 kJ/mol.

The strict selectivity of the P2 transporter for purines over pyrimidines or polyamines (Table 1) shows that constituents of the purine ring are essential for binding to P2. For interaction with the P2 transporter, the most important feature on the purine ring appears to be the amine group at position 6 of adenosine. Purine nucleosides or nucleobases with a substitution at this position displayed low affinity for this transporter (Fig. 4B and Table 1). For example, high concentrations of inosine (>250 μM), identical to adenosine except for the keto rather than amine group at position 6, failed to inhibit P2-mediated adenosine uptake (Fig. 2). However, the P2 transporter does not exhibit a broad permeant specificity.

The polyamines, putrescine and spermidine (Table 1), and amino acids (not shown) had no effect on P2-mediated adenosine transport. The nitrogen residue at position 1 is part of the P2 binding motif, as shown by the relatively high affinity for 2-aminopyridine (K_i = 14 ± 4.9 μM; Fig. 4F), which resembles adenosine only in that it has an amine group in a position ortho from a pyrimidine nitrogen. The ΔG^0 for 2-aminopyridine was 6.8 kJ/mol lower than that for adenosine, indicating that the main binding motif for the P2 transporter is $H_2N—C(R_1)=N—R_2$. Further evidence for the involvement of N1 is that oxopurines like guanosine and inosine are almost exclusively in the keto rather than the enol tautomeric forms (Chenon et al., 1975; Fig. 5), which ensures protonation of N1. It is the loss of hydrogen bonds at both positions 1 and 6 that causes the strict selectivity of aminopurines over oxopurines. The aromaticity of the ring may also contribute to the ligand/transporter interaction (see *Discussion*).

Although the identified motif described above does confer selective high affinity for the P2 transporter, it does not by itself ensure optimal binding of the ligand (compare the submicromolar K_i values for adenine and adenosine with the micromolar K_i value for 2-aminopyridine). Of other elements on the purine ring, N7 and N3 were not directly involved in ligand/transporter interactions because tubercidin and 3-deazaadenosine retained high affinity (Fig. 4, C and E, and Table 1). However, the nitrogen residue at position 9 appears to be important because the K_i value for the adenosine analog formycin A was about 30-fold higher (Fig. 4D) than that for other adenosine analogs. The ΔG^0 for this ligand was 9.1 kJ/mol lower than that for adenosine, less than the loss of an H-bond. Indeed, N9 is incapable of forming H-bonds and must contribute to the ligand binding in a different way (see *Discussion*). The affinity for 8-azidoadenosine was even more reduced (K_i = 330 ± 140 μM), but the effect of the azido group at that position is hard to interpret and may be in part due to steric interference. Finally, substitutions at position 2 may also lead to some loss of affinity, as illustrated by the 6-fold increased K_i value for 2-chloroadenosine (Table 1), possibly as the result of reduced electron density on N1.

Substrate Recognition for H2 Nucleobase Transporter. In addition to the two adenosine transporters de-

TABLE 1

K_i values for potential inhibitors of P1- and P2-mediated adenosine uptake in *T. b. brucei* bloodstream forms

Initial rates of transport were measured by incubating 10^7 cells for 10 s with [^{3}H]adenosine in the presence of increasing amounts of inhibitor (at least 8 points) and 100 μM adenine (P1 mediated) or 100 μM inosine (P2 mediated). Initial rates of uptake were then plotted versus log inhibitor concentrations to determine IC_{50} values, which were converted to K_i values using a K_m value of 0.38 μM for P1 and a K_m value of 0.92 μM for P2.

Compound	P1 Transporter		ΔG^0	P2 Transporter		ΔG^0
	K_i	n		K_i	n	
	μM		*kJ/mol*	*μM*		*kJ/mol*
Purine nucleosides						
Adenosine	0.36 ± 0.05	3	−36.8	0.91 ± 0.29	3	−34.5
Inosine	0.44 ± 0.10	5	−36.3	N.E., 100	3	
Guanosine	1.8 ± 0.3	3	−32.7	N.E., 250	3	
2′-Deoxy-adenosine	0.19 ± 0.02	3	−38.3	0.23 ± 0.04	3	−37.9
3′-Deoxy-adenosine	210 ± 48	2	−21.0	N.D.		
5′-Deoxy-adenosine	100 ± 7	2	−22.7	N.D.		
2′-Deoxy-inosine	0.34 ± 0.11	3	−36.9	170 ± 23	3	−21.5
Tubercidin	78 ± 6.4	3	−23.4	3.8 ± 0.7	3	−30.9
Formycin A	1.7 ± 0.1	3	−32.9	36 ± 6.6	3	−25.3
Formycin B	1.2 ± 0.1	3	−33.8	N.E., 500	3	
3-Deazaadenosine	83 ± 17.1	3	−23.3	0.29 ± 0.06	3	−37.3
2-Chloroadenosine	15 ± 5.3	3	−27.6	9.7 ± 3.4	3	−28.6
8-Azidoadenosine	9.5 ± 2.1	3	−28.6	330 ± 140	3	−19.8
Ribavirin	28 ± 5.9	3	−26.0	N.D.		
Ganciclovir	>250	2		N.D.		
Pyrimidine nucleosides						
Uridine	830 ± 86	2	−18.7	N.E., 500	2	
Thymidine	44 ± 10	3	−11.4	N.E., 500	3	
Cytidine	N.E., 250	3		N.E., 250	3	
Purine nucleobases						
Adenine	N.E., 250	5		0.30 ± 0.02	3	−37.2
Hypoxanthine	>1000	5		>500	3	
Xanthine	>250	4		110 ± 10	3	−22.6
Allopurinol	>500	2		260 ± 62	3	−20.5
Oxypurinol	>1000	2		300 ± 120	3	−20.1
Pyrimidine bases						
Thymine	N.E., 500	2		N.E., 500	2	
Uracil	N.E., 500	3		N.E., 500	3	
5-Fluorouracil	N.E., 500	2		N.E., 500	2	
Transport inhibitors						
NBMPR	143 ± 65	2	−21.9	74 ± 27	2	−23.6
Dilazep	>250	2		>150	2	
Polyamines						
Spermidine	N.E., 250	2		N.E., 250	2	
Putrescine	N.E., 250	2		N.E., 250	2	
Other						
2-Aminopyridine	N.D.			14 ± 4.9	3	−27.6
Ribose	N.E., 1000	2		N.D.		

N.E., no effect, meaning <10 percent stimulation or inhibition of transport at the indicated concentration (μM).
N.D., not determined.

scribed here, bloodstream forms of *T. b. brucei* express at least two additional purine transporters with hypoxanthine as their main substrate (De Koning and Jarvis, 1997b), designated H2 and H3. The H2 transporter seems to be the more important because this carrier mediates more than 80% of hypoxanthine flux at physiological concentrations, recognizes a wider array of substrates, and has a higher affinity for all purine nucleobases (De Koning and Jarvis, 1997b). In light of the identification of the substrate-binding motifs for the adenosine transporters and the importance of establishing the affinities of the various transporters for diamidine and arsenical drugs (see later), we reanalyzed the selectivity profiles of the H2 transporter to identify structures that confer high affinity as far as this can be done with the available data (Table 2).

In contrast to the P2 transporter, the H2 transporter was preferentially inhibited by oxopurines, with the inhibition constant for adenine almost 30-fold less than the K_m value for hypoxanthine influx (Table 2). This implicates either the 6-keto group as an H-bond acceptor or the N(1)H as an H-bond donor. The difference in binding energy of just 8 kJ/mol between hypoxanthine and adenine was inconsistent with both groups being involved in binding. The more likely explanation seems to be a hydrogen bond to the 6-keto group because the H2 transporter displayed a 3-fold lower affinity for guanine compared with hypoxanthine. The involvement of a N(1)H H-bond also seems unlikely because the introduction of an amine substitution at position 3 might have strengthened rather than weaken the interaction by withdrawing electron density from N1.

Another important feature for H2 recognition appeared to be N7 of the imidazole ring because the shift of this residue to position 8 caused substantial loss of binding energy (8.6 kJ/mol, allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] versus hypoxanthine). This effect is not due to tautomeric shifts on the pyrimidine ring (allopurinol, like hypoxanthine, being predominantly in the lactam rather than lactim state; Hänggi et al., 1993). Similarly, the loss of binding energy for xanthine (10.6 kJ/mol), compared with hypoxanthine, is most likely due to the predominance of the N(7)H tautomer for xanthine, which is more stable than the N(9)H tautomer by 9.2 kJ/mol (Rastelli et al., 1997). Hypoxanthine is more stable in the N(9)H tautomeric isoform, by about the same amount of energy (Rastelli et al., 1997). Finally, the preference of this transporter of nucleobases over nucleosides shows that bulky substitutions on N9 are unfavorable for binding.

Diamidines and Melaminophenylarsenicals Display High Affinity for P2 Transporter. Alignments of the above ligand recognition profiles with the structural formulae of diamidines such as pentamidine and diminazene aceturate (berenil) and melaminophenylarsenicals such as melarsoprol (see Fig. 5) would predict that these trypanocides have little affinity for the P1 and H2 transporters but may have a high affinity for the P2. To test this hypothesis, we examined these drugs as inhibitors of [^{3}H]adenosine and H2-mediated [^{3}H]hypoxanthine uptake.

The results depicted in Table 3 show that these predictions were upheld. The P2 transporter displayed high affinity (K_i = 0.4–2.4 μM) for all three drugs, whereas the other transporters displayed affinities that were typically 2 orders of magnitude lower. One exception was the H2 transporter, which exhibited a relatively high binding affinity for melarsoprol (K_i = 10.1 ± 2.6 μM), perhaps as a result of the structural similarity between the melamine group and adenine (K_i 3.2 ± 1.1 μM).

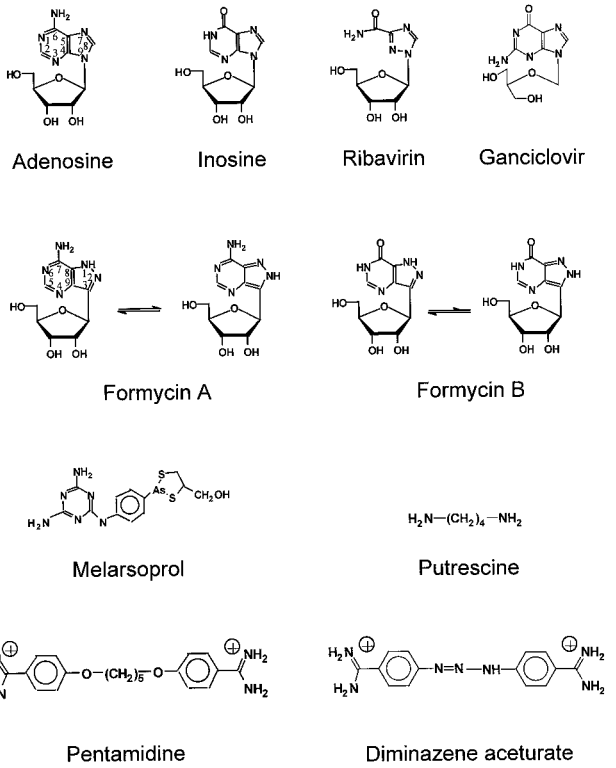

Fig. 5. Structural formulas of selected purine (analogs) and trypanocides. Care has been taken to depict the dominant tautomeric forms. Formycins A and B are both 85% in the N(1)H tautomeric form and 15% in the N(2)H form (Bzowska et al., 1992). Inosine, adenosine, and guanosine do not exhibit appreciable tautomerism (Chenon et al., 1975; Bzowska et al., 1992). Note the different IUPAC numbering for purines and the pyrazolo[4,3-*d*]pyrimidine ring of the formycins.

Discussion

In this study, we confirmed the presence of two high-affinity adenosine transporters, designated P1 and P2 (Carter and Fairlamb, 1993), in *T. b. brucei* bloodstream forms. No evidence was obtained from the inhibitor dose-response curves of either P1- or P2-mediated adenosine transport for

TABLE 2
Rank order of inhibitors of the H2 nucleobase transporter of *T. b. brucei* bloodstream forms

Compound	K_i	ΔG^0
	μM	*kJ/mol*
Hypoxanthine	0.12 ± 0.02	−39.4
Guanine	0.36 ± 0.18	−36.7
Adenine	3.2 ± 1.1	−31.3
Allopurinol	4.0 ± 2.2	−30.8
Xanthine	8.8 ± 3.9	−28.8
Guanosine	10.9 ± 1.8	−28.3
Oxypurinol	15.7 ± 3.5	−27.4
Uracil	60 ± 14	−24.1
Thymine	82 ± 25	−23.3
Inosine	167 ± 58	−21.5
Adenosine	590 ± 175	−18.4

K_i values were obtained from De Koning and Jarvis (1997b).

the presence of additional transporters contributing significantly to the influx of adenosine. For example, the Hill slopes were not significantly different from −1, and 100% inhibition was consistently observed. On the basis of inhibition data, the binding energies for the interactions of various ligands for either transporter were determined, and models were proposed for the interactions between permeant binding site and adenosine. P1 and P2 differed in their ligand recognition profiles, indicating that that the permeant binding pocket for the two adenosine transporters are distinguishable.

The P1 transporter displayed a high affinity for all natural purine nucleosides ($K_i \sim 1$ μM) and most purine nucleoside analogs tested, whereas P2 recognized only adenosine nucleosides. In addition, the P1 transporter was completely insensitive to inhibition by nucleobases, but P2 displayed a higher affinity for adenine than for adenosine (affinity constant of 0.3 versus 0.9 μM). Analysis of the inhibition profiles allowed the identification of the structures within the adenosine molecule that engage in the binding of adenosine to the transporter. For the P1 transporter, the loss of 13.4 to 15.8 kJ/mol in Gibbs free energy when N3, N7, 3′OH, or 5′OH is replaced by carbon or hydrogen residues identifies each of these groups as a potential acceptor (the nitrogen residues) or donor of a hydrogen bond. The bonding energy of adenosine was determined to be −36 kJ/mol, whereas the energy of the four H-bonds is predicted to be −56 kJ/mol. This apparent difference suggests considerable cooperativity between the bonds and indicates a reason for the failure of ribose and hypoxanthine to inhibit adenosine uptake via the P1 transporter, even though each compound has two of four essential residues to form H-bonds within the putative permeant binding site.

The region of adenosine most involved in interaction with the P2 transporter was identified as N(1)—C(6)—NH_2, with N1 acting as a potential H-bond acceptor and the amine group a possible donor of one or two H-bonds. Cooperation between these bonds would result from the withdrawal of electron density from the amine group through the formation of the H-bond to N1. The importance of this structure in ligand/transporter interaction was demonstrated most convincingly by the inhibitory effect of 2-aminopyridine on P2-mediated adenosine uptake and fully explains the total preference for 6-aminopurines over oxopurines, as the latter molecules, possessing a protonated N1, have a completely reversed H-bond donor/acceptor pair on positions 1 and 6. However, the H-bonding to the above-mentioned structure is not sufficient to account for the −34.5 kJ/mol in binding energy between the P2 transporter and adenosine. The similar binding energies of 2-aminopyrimidine and formycin A, about 8 kJ/mol lower than that for adenosine, identifies N9 as essential for high-affinity binding. The lone pair of electrons at N9 would be mostly fed into the π-system of the pyrimidine ring and be unavailable for H-bonding to the transport protein, thereby creating a partial positive charge on N9 and making the π-system more electron rich. In the environment of a hydrophobic binding pocket, either might be significant for substrate/carrier binding. Apart from electrostatic attractions, π-π interactions between aromatic rings can contribute up to 10 kJ/mol to the binding energy (Hunter et al., 1991). The possibility of π-π interactions significantly contributing to ligand binding by the P2 transporter suggests that an aromatic residue may play a significant role in P2/ligand interactions as shown for hypoxanthine/guanine/xanthine phosphoribosyltransferases from a variety of sources (Eads et al., 1994; Schumacher et al., 1996; Somoza et al., 1996; Vos et al., 1998). Koellner et al. (1998) also concluded that in *Escherichia coli* purine nucleoside phosphorylase, aromatic interactions between the purine ring and Phe159/Tyr160 direct the base into its binding position.

TABLE 3

K_i values for selected trypanocides

Uptake of 20 nM [^{3}H]adenosine and 30 nM [^{3}H]hypoxanthine by *T. b. brucei* bloodstream forms was performed as described in the legend to Table 1.

Trypanocide	P1	*n*	P2	*n*	H2	*n*
Melarsoprol	33.1 ± 0.6	3	0.54 ± 0.15	3	10.1 ± 2.6	3
Pentamidine	32.2 ± 3.6	3	0.43 ± 0.02	3	24.9 ± 6.5	3
Diminazene aceturate	176 ± 33	4	2.36 ± 0.46	3	102 ± 17	3

TABLE 4

Comparison of the functional properties of nucleoside transporter subtypes in bloodstream forms of *T. b. brucei* and mammalian cells

	Trypanosomes[a]		Mammalian Cells[b]			
			Equilibrative		Na^+ Dependent	
	P1	P2	*es*	*ei*	N1	N2
Na^+ dependent	No	No	No	No	Yes	Yes
H^+ dependent	Yes	Yes	No	No	No	No
Permeant/inhibitor[c]						
Adenosine	++++	++++	++	++	+++/++	+++/++
Inosine	++++	−	++	++	+++/++	−
Guanosine	+++	−	++/+	+	+++/++	−
Formycin B	+++	−	+	+	++	−
Tubercidin	++	+++	+	+	−	++[d]
Thymidine	++	−	++/+	++/+	−	+++/++
Uridine	+	−	++/+	++/+	+++/++	+++/++
Nitrobenzylthioinosine	No	No	Yes	No	No	No

[a] Data from the present study and Carter and Fairlamb (1993) and De Koning et al. (1998).
[b] Summarized from recent review articles by Griffith and Jarvis (1996) and Cass (1995) and the references therein.
[c] Potency expressed in the following manner: ++++, K_m/K_i <1 μM; +++, K_m/K_i 1–10 μM; ++, K_m/K_i 10–100 μM; and +, K_m/K_i >100 μM.
[d] Inhibits but is not transported.

Thus, optimal binding to the P2 transporter requires 1) an amidine group $H_2N—C(R_1)=N—R_2$, that may be integrated into a pyridine or pyrimidine ring; 2) an aromatic system associated or integrated with the amidine group; and 3) an electronegative group attached to the aromatic ring, para to the amidine, that is able to contribute to the π-system with a lone electron pair. These results are entirely in agreement with predictions from an earlier study (E. Akuffo and A. H. Fairlamb, unpublished results) based on the ability of various purines and other molecules to abrogate melarsen-oxide-induced lysis of *T. b. brucei*. In addition, the criteria for P2 recognition are all met by the diamidine drugs pentamidine and berenil, as well as by the melaminophenylarsenicals (Fig. 5). The melamine ring of melarsoprol by itself fulfills all the requirements, and the relatively large phenylarsenical group does not interfere with the high-affinity binding to the P2 transporter. The finding that pentamidine showed equally high affinity for this transporter suggests that the amidine group does not need to be integrated in the aromatic ring and that an ether group on the para position is equally effective as an amine in enhancing binding. The binding energy for berenil was somewhat lower than that of the other two drugs, possibly as a result of its less flexible structure.

The interaction of melaminophenylarsenicals and diamidines with the P2 transporter could be due to the binding of these compounds to the permeation site, without necessarily being transported into the cell. However, earlier studies have shown that both adenosine and adenine, but not inosine, protected bloodstream forms of trypanosomes from melarsen-oxide-induced lysis in vitro (Carter and Fairlamb, 1993). This result was consistent with melaminophenylarsenicals entering trypanosomes via the P2 transporter, and this notion was further supported by the observation that P2 transporter activity was absent in a melarsoprol-resistant strain (Carter and Fairlamb, 1993). In subsequent studies, a P2-like transporter has also been implicated in the uptake of berenil in *Trypanosoma equiperdum* (Barrett et al., 1995), of cymelarsan in *Trypanosoma evansi* (Ross and Barns, 1996), and of pentamidine in *T. b. brucei* (Carter et al., 1995). Thus, we conclude that the above drugs are specific permeants for the P2 transporter and the structural rationale for the high-affinity interaction of these compounds with the P2 transporter have been defined in this study.

The identification of those structures that are essential for high-affinity binding with the trypanozoon transporters enables the selection or design of novel trypanocides that will be taken up with high efficiency. Because all the *T. b. brucei* nucleoside and nucleobase transporters investigated to date are proton symporters (De Koning and Jarvis, 1997a,b, 1998; De Koning et al., 1998), these substrates should be efficiently concentrated intracellularly. However, for such compounds to be therapeutically relevant, they should also display selectivity for the parasite transporters over the corresponding host transporters. Two equilibrative (designated *ei* and *es*) and five concentrative nucleoside (designated N1–N5) transporters have been identified in mammalian cells, with substrate affinities for naturally occurring nucleosides of 10 to 300 μM and 5 to 50 μM, respectively (for a review, see Griffith and Jarvis, 1996). Not only are these affinities one to two orders of magnitude lower than those of the *T. b. brucei* purine transporters (Tables 1 and 4; De Koning and Jarvis, 1997a,b, 1998), the substrate recognition profiles of the mammalian transporters are also significantly different. For example, all the mammalian transporters for purine nucleosides recognize at least some pyrimidine nucleosides (Griffith and Jarvis, 1996) and require a ribose moiety for transport, whereas the *T. b. brucei* purine transporters do not bind pyrimidines and the affinity for the P2 transporter is actually slightly reduced by the presence of a ribose moiety. On this basis, the prospect of selective uptake of toxic chemotherapeutic compounds by the P1 and P2 transporters of trypanosomes seems hopeful. The *T. b. brucei* transporters, particularly P1, would recognize cytotoxic nucleosides, such as formycin A, ribavirin, and tubercidin, at therapeutic levels (Table 1), whereas the mammalian transporters generally have low affinity for such agents (Griffith and Jarvis, 1996; Jarvis et al., 1998). Indeed, it was recently demonstrated by Tye et al. (1998) that a melamine moiety can be used to deliver potentially cytotoxic polyamine analogs to trypanosomes. However, these compounds, although inhibitors of P2-mediated adenosine uptake, showed only weak trypanocidal activity in vitro.

In conclusion, the different pattern of binding forces involved in P1 and P2 transporter/substrate interactions suggests that their binding pockets may be quite dissimilar. With the recent cloning of the mammalian nucleoside transporters, a number of studies have appeared using chimeric transporters or site-directed mutants that have started to define the essential residues on the equilibrative and active nucleoside carriers for transport selectivity or inhibitor sensitivity (Wang and Giacomini 1999a,b; Sundaram et al., 1998). The present study approaches this problem from the substrate rather than the protein side and has resulted in formulating a structure-activity relationship for the binding of adenosine to the P1 and P2 tansporters. With efforts to clone the *T. b. brucei* nucleoside transporters under way, these transporters should provide excellent models to further study transporter structure and mechanism.

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