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Uptake of Pentamidine in *Trypanosoma brucei brucei* is Mediated by Three Distinct Transporters: Implications for Cross-Resistance with Arsenicals

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ABSTRACT

The trypanocidal action of pentamidine is dependent on the rapid, selective accumulation of this drug by the parasite. We have investigated pentamidine transport by the bloodstream and procyclic life cycle stages of *Trypanosoma brucei brucei*. In bloodstream forms, 50 to 70% of [^{3}H]pentamidine was transported by an adenosine-sensitive pentamidine transporter (ASPT1) that displayed a K_m value of 0.26 ± 0.03 μM and K_i values of 0.45 ± 0.04 and 2.5 ± 0.8 μM for adenine and berenil, respectively. These values are very similar to those for inhibition of [^{3}H]adenosine uptake by the P2 adenosine/adenine transporter, suggesting that ASPT1 and P2 may be identical. The remaining 30 to 50% of [^{3}H]pentamidine transport was mediated by a low-capacity high-affinity pentamidine transporter (HAPT1) and a high-capacity low-affinity pentamidine transporter (LAPT1), with K_m values of 36 ± 6 nM and 56 ± 8 μM, respectively. HAPT1 was inhibited by propamidine but displayed only low affinity to berenil and stilbamidine, whereas LAPT1 was not inhibited by any of these diamidines. Neither transporter was inhibited by melarsen oxide. In procyclics, an HAPT1-analog (procyclic pentamidine transporter; PPT1) was characterized, but no adenosine-sensitive pentamidine transport could be detected. Treatment with ionophores revealed that PPT1 may be a proton/pentamidine cotransporter.

Diamidines, including pentamidine and berenil (diminazene aceturate), have been first-line drugs for the treatment of early stage African trypanosomiasis for decades (Pépin and Milord, 1994). While their mechanism of action remains a matter of debate, it is generally accepted that their selective toxicity for the parasite is the result of accumulation by the trypanosome, but not the host cell, to very high intracellular levels (Damper and Patton, 1976a; Carter et al., 1999). Damper and Patton (1976a,b) first reported that the uptake of pentamidine by *Trypanosoma brucei brucei* bloodstream forms is mediated by high-affinity transporters that are energy-dependent and competitively inhibited by other diamidines. Carter et al. (1995) tentatively identified the pentamidine carrier as the P2 adenosine/adenine transporter that has also been linked to the uptake of the melaminophenyl arsenical class of trypanocides (Carter and Fairlamb, 1993; Mäser et al., 1999; De Koning et al., 2000a). The involvement of the P2 transporter in diamidine uptake was also inferred from the observation that a berenil-resistant clone of *T. equiperdum* expressed a P2 transporter with much reduced affinity (Barrett et al., 1995). A biochemical basis for the high-affinity transport of such diverse molecules as adenosine, pentamidine, and melarsoprol by the same transporter was provided by the identification of the substrate recognition motif for P2 (Barrett and Fairlamb, 1999; De Koning and Jarvis, 1999), which is present on both classes of drugs.

However, significant problems with the hypothesis of pentamidine uptake by the P2 transporter remained. A 10-fold excess of adenosine failed to inhibit [^{3}H]pentamidine uptake by *T. brucei brucei* (Carter et al., 1995), and a cloned *T. brucei brucei* transporter with P2-like activity, TbAT1, conferred sensitivity to arsenicals when expressed in *Saccharomyces cerevisiae*, but it was not inhibited by pentamidine (Mäser et al., 1999). In addition, the *T. brucei brucei* clone RU15, which did not express measurable P2 transport activity (Carter and Fairlamb, 1993), was resistant to melaminophenyl arsenicals and berenil, but not to pentamidine (Fairlamb et al., 1992). Indeed, it appears that clinical and veterinary isolates as well as laboratory strains refractory to melaminophenyl arsenicals are usually highly cross-resistant to berenil but less so to pentamidine (Bacchi, 1993). Treatment with pentamidine of early stage African sleeping sickness is not endangered by resistance, although resistance to melarsoprol is a

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ABBREVIATIONS: ASPT1, adenosine-sensitive pentamidine transporter-1; CCCP, carbonyl cyanide chlorophenylhydrazone; HAPT1, high-affinity pentamidine transporter-1; LAPT1, low-affinity pentamidine transporter-1; PPT, procyclic pentamidine transporter; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide.

rapidly growing problem (Legros et al., 1999; Kaminsky and Mäser, 2000). These observations led to the hypothesis that, while berenil and melaminophenyl arsenicals share a common transporter, pentamidine is either taken up by a different transporter or by several different transporters, possibly including the P2. In the current study, this hypothesis was tested by a thorough analysis of [^{3}H]pentamidine uptake by *T. brucei brucei*.

Experimental Procedures

Materials. [^{3}H]Pentamidine isethionate was synthesized by Amersham Pharmacia Biotech (3.63 TBq/mmol) for Dr. M. P. Barrett (University of Glasgow) and generously donated for this study. [2,8,5′-^{3}H]Adenosine was from NEN Life Science Products (2.0 TBq/mmol). Most test compounds were purchased from Sigma (St. Louis, MO), including CCCP, nigericin, berenil, and pentamidine (also obtained from Rhône-Poulenc Rorer, Montrouge, France). Melarsen oxide, stilbamidine, and propamidine were generous gifts from Rhône-Poulenc.

Trypanosomes. *T. brucei brucei* strain 427 bloodstream trypanosomes were taken from frozen stocks and injected in adult female Wistar rats. Blood was collected at peak parasitemia by cardiac puncture under anesthesia. Parasites were isolated from the blood on a DE52 (Whatman, Maidstone, UK) column (Lanham, 1968) and 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) by centrifugation at 2000 rpm. Cells were counted using a hemocytometer and resuspended in assay buffer at 10^8 cells/ml. Procyclic *T. brucei brucei* were cultured in SDM79 culture media as described (De Koning et al., 1998) and washed into assay buffer as described above. At the end of each experiment, cell motility was checked under a phase-contrast microscope.

Transport Studies. Transport of [^{3}H]pentamidine and [^{3}H]adenosine was performed as previously described with minor modifications (De Koning and Jarvis, 1997a,b, 1999; De Koning et al., 1998). Briefly, 100 μl of assay buffer containing radiolabel and, where appropriate, test compound in assay buffer was layered over 250 μl of 7:1 mixture of di-*n*-butylphthalate (BDH, Poole, Dorset, UK) and light mineral oil (Sigma) in a microfuge tube and mixed with 100 μl of trypanosomes (10^7 cells, in assay buffer). Incubations (between 10 and 60 s) were performed at 22 or 0°C and stopped by the addition of 1 ml of ice-cold 1 mM unlabeled permeant in assay buffer and centrifugation through the oil (13,000 rpm, 30 s). Tubes were then flash frozen in liquid nitrogen and the bottom (containing the cell pellet) cut off with a tube cutter. This was collected in a scintillation tube, incubated for 30 min with 250 μl of 2% SDS, mixed with 3 ml of Ecoscint A (National Diagnostics, Atlanta, GA), and shaken well. After incubation overnight at room temperature, radioactivity was determined by scintillation counting. Experiments under sodium-free conditions were performed as described in sodium-free media containing *N*-methyl-D-glucamine (De Koning et al., 1998). When permeabilized cells were used, trypanosomes were placed on ice for 5 min at 5×10^8 cells ml^{-1} and incubated with 500 μg ml^{-1} of lysolecithin for 1 min before 10-fold dilution with assay buffer. Cells were then washed and resuspended in fresh assay buffer at 10^8 cells ml^{-1} and used for experiment as usual.

All experiments were carried out in triplicate or more, and kinetic parameters were determined a minimum of three times in independent experiments and given as average and S.E. IC_{50}, V_{max}, and K_m values were determined using the FigP (Biosoft, Ferguson, MO) and Prism (GraphPad, San Diego, CA) software packages. K_i values were determined using the Cheng and Prusoff (1973) equation: $K_i = IC_{50}/[1 + (L/K_m)]$, where L is the permeant concentration and IC_{50} values were determined from full dose-response curves with a minimum of eight points spread over the relevant range. Hill coefficients were always close to −1, consistent with monophasic competitive inhibition, except where indicated. In these cases, data were fitted to equations for monophasic and biphasic inhibition in the Prism software package and the two fits compared with an F-test. Data were considered to fit a two-phase inhibition better when $P < 0.05$.

In all cases, transport of permeant is understood to mean mediated transport, after subtraction of radiolabel entering the cell by diffusion. Diffusion was taken to be the difference in uptake between an incubation of cells in the presence of 1 mM unlabeled permeant at room temperature for the same length of time as the rest of the assay, and a similar incubation with both the cells and permeant kept on ice, stopped with stop solution immediately after mixing, and spun through oil. Rates of uptake were calculated from $V = (V_{max} \times L)/(L + K_m)$.

Results and Discussion

Characteristics of [^{3}H]Pentamidine Uptake in Bloodstream Forms of *T. brucei brucei*. Uptake of 25 nM [^{3}H]pentamidine in bloodstream forms of *T. brucei brucei* was rapid, and linear for at least 3 min, with a rate of 0.0045 ± 0.0004 pmol (10^7 cells)$^{-1}$ s^{-1} (Fig. 1A). In the presence of 250 μM unlabeled pentamidine, the rate was reduced by 97%, indicating that the vast majority of pentamidine uptake is through a saturable, carrier-mediated process, with only a very minor diffusion component. Adenosine (250 μM) inhibited 25 nM [^{3}H]pentamidine uptake by 65% (Fig. 1A). Closer examination revealed that adenosine consistently and dose dependently inhibits 50 to 70% of mediated pentamidine transport, with a K_i value of 0.80 ± 0.12 μM (n = 14). Figure 1B shows a typical experiment in which adenosine inhibited ~50% of mediated [^{3}H]pentamidine (25 nM) transport with a K_i value of 1.1 μM. In the same experiment, adenine inhibited pentamidine transport to a similar degree, with a K_i value of 0.42 ± 0.04 μM (n = 5) (Fig. 1B). The K_i values of adenosine and adenine on [^{3}H]pentamidine transport are very similar to those for adenosine and adenine on [^{3}H]adenosine transport mediated by the P2 transporter (0.91 ± 0.29 and 0.30 ± 0.02 μM, respectively) (Carter and

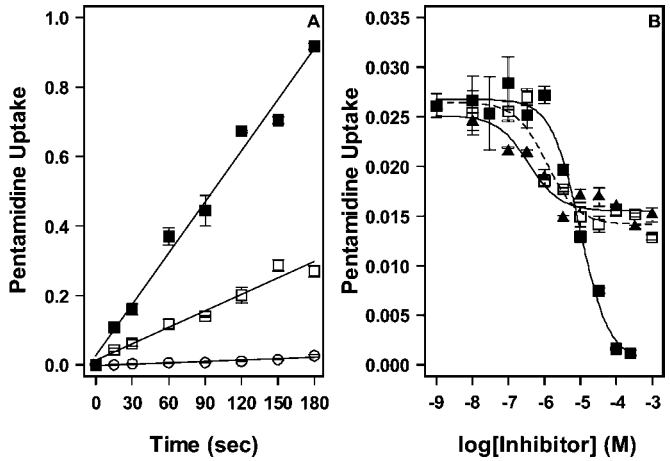

Fig. 1. Effect of adenosine and adenine on pentamidine transport in *T. brucei brucei* bloodstream forms. Units for pentamidine uptake are pmol (10^7 cells)$^{-1}$ s^{-1}. A, uptake of 25 nM [^{3}H]pentamidine was measured over various intervals in the presence of 250 μM adenosine (□) or 250 μM unlabeled pentamidine (○). Control cells were incubated with the same volume of assay buffer without inhibitor (■). B, inhibition of 25 nM [^{3}H]pentamidine by various concentrations of adenosine (□, dashed line), adenine (▲, solid line), and pentamidine (■, solid line). The level of inhibition was 47, 42, and 98%, respectively, and K_i values for this experiment were 1.1 μM (adenosine) and 0.33 μM (adenine).

Fairlamb, 1993; De Koning and Jarvis, 1999; De Koning et al., 2000a). It thus appears that pentamidine transport in *T. brucei brucei* bloodstream forms is mediated by an adenosine-sensitive pentamidine transporter (ASPT1), which may be identical to P2, as well as by one or more adenosine-insensitive transporters.

To further characterize these transporters, K_m values for [^{3}H]pentamidine were obtained by measuring transport of 12.5 nM [^{3}H]pentamidine in the presence of variable amounts of unlabeled pentamidine. To separate adenosine-sensitive and -insensitive transport, this procedure was performed both in the presence and absence of 250 μM adenosine. Subtraction of adenosine-insensitive uptake from the total uptake yielded the adenosine-sensitive uptake (Fig. 2). The adenosine-insensitive uptake was best described by a two-transporter model ($P < 0.001$), indicating the presence of a high-affinity pentamidine transporter (HAPT1) and a low-affinity pentamidine transporter (LAPT1). In contrast, the adenosine-sensitive uptake yielded a curve with an IC_{50} value of 0.67 μM and a Hill coefficient near -1 ($n = 3$) and was entirely consistent with a one-transporter (ASPT1) model (Fig. 2).

K_m and V_{max} values (Table 1) were obtained by converting the data in Fig. 2 to hyperbolic Michaelis-Menten plots. The K_m value for ASPT1 was very similar to K_i values of pentamidine for P2-mediated adenosine transport reported previously [0.48 μM (Carter et al., 1995); 0.43 $\pm$ 0.02 μM (De Koning and Jarvis, 1999)]. While HAPT1 displayed a very high affinity for pentamidine, it also has a very low capacity, and the order for both K_m and V_{max} values is HAPT1 $<$ ASPT1 $<$ LAPT1.

The determination of accurate K_m and V_{max} values for the three transporters allowed the prediction of the amount of [^{3}H]pentamidine that will be transported by any one of these at a given extracellular pentamidine concentration. Because of the combination of higher capacity than HAPT1 and much higher affinity than LAPT1, ASPT1 is predicted to mediate 55 to 80% of the total uptake from nanomolar to lower micromolar concentrations. At 10 nM, HAPT1 mediates 85% of the adenosine-insensitive pentamidine uptake, whereas at 2 μM pentamidine, 87% of adenosine-insensitive uptake is through LAPT1. This allows the adenosine-insensitive transporters to be studied in near isolation, depending on the [^{3}H]pentamidine concentration. Plasma concentrations in patients treated with the standard 4 mg/kg dosing have been determined at 0.5 to 2.5 μM (Waalkes and DeVita 1970; Waalkes et al., 1970). Over this range, the contribution of LAPT1 increased from 13 to 35% of total pentamidine uptake due to its very high capacity.

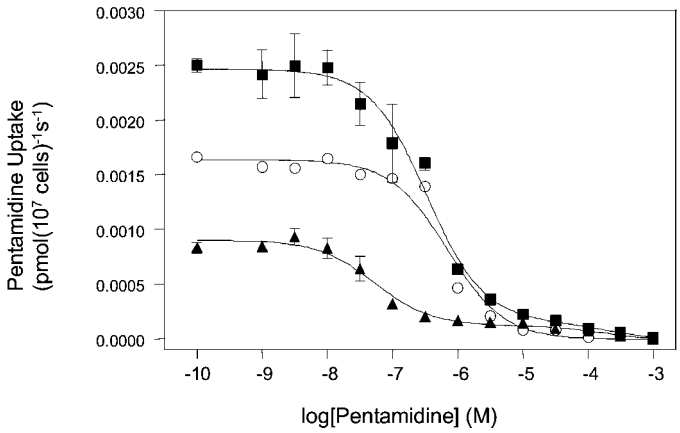

Fig. 2. Uptake of [^{3}H]pentamidine by bloodstream *T. brucei brucei* is mediated by three transporters. Uptake of 12.5 nM [^{3}H]pentamidine in the presence (▲) or absence (■) of 250 μM adenosine and various concentrations of unlabeled pentamidine represents adenosine-insensitive pentamidine uptake and total pentamidine uptake, respectively. Adenosine-sensitive uptake (○) was derived from these data by subtracting the adenosine-insensitive uptake from the total uptake. The graph shows the average of triplicate determinations and one of several identical experiments.

Attempts were made to identify the physiological substrate of the HAPT1 transporter. Up to 1 mM hypoxanthine did not inhibit uptake of 25 nM [^{3}H]pentamidine in the presence of 250 μM adenosine (Fig. 3). Other potential substrates that similarly failed to inhibit HAPT1-mediated pentamidine transport included inosine, adenine, uracil, uridine, sodium pyruvate (all at 2.5 mM), the amino acids arginine, lysine, glutamine, asparagine, phenylalanine, tryptophan (all at 1 mM), and tyrosine (50 μM), the dipeptide Arg-Lys, the polyamines putrescine, spermidine, and spermine, biotin, folic acid, *para*-aminobenzoic acid, and thiamine (all at 1 mM). Each of these compounds was tested in at least three independent experiments, in triplicate, at a [^{3}H]pentamidine concentration of 25 nM, and none produced a statistically significant difference ($P < 0.05$ in an unpaired Student's t test) from controls with no inhibitor.

The failure of purines and pyrimidines to inhibit 12.5 nM [^{3}H]pentamidine uptake in *T. brucei brucei* bloodstream forms or procyclics proves that none of the hypoxanthine or uracil transporters (De Koning and Jarvis, 1997a,b, 1998) are involved in this process, even though pentamidine does inhibit hypoxanthine uptake in *T. brucei brucei* bloodstream forms, with a K_i value of 25 $\pm$ 7 μM (De Koning and Jarvis, 1999). High-affinity pentamidine uptake was also not mediated by polyamine transporters, as has been reported for *Leishmania* spp. (Basselin et al., 1997). Inhibition of amino acid transport by pentamidine has been reported in *Crithidia fasciculata* (Gutteridge, 1969) and *Leishmania* spp. (Basselin et al., 1997), although the latter effect was found to be noncompetitive. Similar experiments to those described here, but using [^{125}I]iodopentamidine as permeant, were performed for LAPT1-mediated pentamidine uptake (De Koning and Jarvis, unpublished observations).

Affinity of the Pentamidine Transporters for Other Trypanocides. The affinity of the various pentamidine transporters for selected other trypanocides was investigated (Table 1). HAPT1 was inhibited by propamidine but displayed little (berenil and stilbamidine) or no (melarsen oxide) affinity for other antitrypanosome drugs (Fig. 3), and it is not expected to play a role in the uptake of these drugs at therapeutic levels. Similarly, LAPT1 was not inhibited by any of these drugs, unless very high concentrations were used (Table 1). ASPT1, in contrast, was strongly inhibited by melarsen oxide (Fig. 3), berenil, stilbamidine, and propamidine. As shown above for adenine, adenosine, and pentamidine, the ASPT1 K_i values for berenil and melarsen oxide were almost identical to those shown for the P2 transporter (Table 1).

Taken together, all available data are consistent with ASPT1 being identical to P2. The amount of [^{3}H]pentamidine transport inhibited by adenosine varied between experiments. Within a single experiment, however, uptake was inhibited to a very similar degree by diverse P2 substrates,

such as adenosine, adenine, melarsen oxide, and various diamidines. As ASPT1 was not inhibited by inosine, the adenosine-sensitive pentamidine uptake was not mediated by the P1 transporter, the only other adenosine carrier in bloodstream trypanosomes (Carter and Fairlamb, 1993; De Koning and Jarvis, 1999). Thus, the substrate recognition profile of ASPT1 is qualitatively and quantitatively indistinguishable from P2.

Pentamidine Uptake in Procyclic *T. brucei brucei*. In view of the above results, as well as of previous studies with diamidine-resistant trypanosomes (see *Introduction*), it was deemed very likely that ASPT1 is identical to the P2 aminopurine transporter. One prediction from this hypothesis was that ASPT1 would not be present in procyclic forms of *T. brucei brucei*, since the P2 transporter is not expressed in this life cycle stage (De Koning et al., 1998, 2000b). This prediction was borne out with the observation that transport of 25 nM, 250 nM, or 1 μM [^{3}H]pentamidine by procyclic *T. brucei brucei* was not inhibited by up to 1 mM adenosine or adenine (not shown). Transport was saturable and was linear for up to 45 min at 1 μM [^{3}H]pentamidine with a rate of 4.4 pmol $(10^7$ cells$)^{-1}$, whereas at 50 nM uptake proceeded only slightly slower [3.2 pmol $(10^7$ cells$)^{-1}$] and was linear until all radiolabel was taken up (Fig. 4A). This result indicates that, in *T. brucei brucei* procyclics and at 50 nM [^{3}H]pentamidine, uptake was already proceeding at near V_{max}, consistent with a single high-affinity transport system rather than the more complex situation in bloodstream forms.

Uptake of 12.5 nM [^{3}H]pentamidine was consistent with a one-transporter model and displayed saturable Michaelis-Menten kinetics with a K_m value of 29 $\pm$ 3 nM and a V_{max} value of 0.039 $\pm$ 0.008 pmol $(10^7$ cells$)^{-1}$ s^{-1} (n = 8). The very high affinity of this PPT1 is not statistically different from the K_m value of HAPT1. Moreover, the affinity of the PPT1 and HAPT1 transporters for other diamidines was also very similar (Table 1). Dixon plots showed the inhibition of PPT1 by propamidine (Fig. 4B) and by berenil (not shown) to be competitive. The main difference between the high-affinity transporters in procyclic and bloodstream *T. brucei brucei* was the V_{max} value, with the procyclic trypanosomes displaying a much higher rate of uptake, probably as a result of a higher level of transporter expression in this life cycle stage.

A low-affinity pentamidine transporter (PPT2) also appeared to be present in procyclics, but no attempt at further characterization of this carrier was made, and at <100 nM [^{3}H]pentamidine concentrations this transporter did not contribute significantly to the pentamidine uptake. As with HAPT1-mediated pentamidine uptake, a range of inhibitors was tested in the hope of identifying the physiological substrate of PPT1. Inosine, putrescine, spermine, spermidine, biotin, folic acid, *para*-aminobenzoic acid, and thiamine were all ineffective at 1 mM. Uracil, lysine, arginine, proline (all at 10 mM), sphingosine (250 μM), pyruvate (2.5 mM), and 250 μM concentrations of the peptides Arg-Lys, Arg-Phe, Lys-Lys-Lys, Arg-Ser-Arg, and $(Lys)_4$ similarly failed to inhibit uptake of 12.5 nM [^{3}H]pentamidine in *T. brucei brucei* procyclics (n = 3). While it is impossible to conclude whether PPT1 and HAPT1 are identical without cloning both transporters, they are certainly very similar and may represent a single gene product expressed in both life cycle stages. The mechanism of

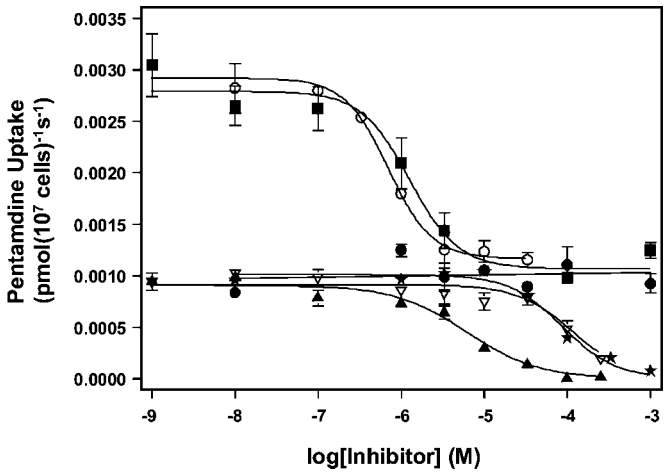

Fig. 3. Effects of trypanocides and hypoxanthine on 12.5 nM [^{3}H]pentamidine uptake by *T. brucei brucei* bloodstream forms. [^{3}H]pentamidine uptake was determined in the presence of various concentrations of adenosine (■, IC_{50} = 1.2 μM) or melarsen oxide (○, IC_{50} = 0.7 μM), as well as in the presence of 250 μM adenosine and increasing concentrations of hypoxanthine (●), propamidine (▲, IC_{50} = 6.1 μM), stilbamidine (▽, IC_{50} = 110 μM), and berenil (★, IC_{50} = 78 μM).

TABLE 1

Kinetic parameters of pentamidine transporters in *T. b. brucei* and K_i values for purines and trypanocides (μM)

Bloodstream form or procyclic *T. b. brucei* was incubated with [^{3}H]pentamidine as described under *Experimental Procedures*. Permeant concentrations were 12.5 nM (PPT1 and HAPT1), 1 μM (LAPT1), 12.5 or 25 nM (ASPT1), or 20 nM (P2). Results are expressed as the mean $\pm$ S.E. of at least three independent expts.

	PPT1[a]	HAPT1[a]	LAPT1[a]	ASPT1[a]	P2[b]
Kinetic Parameters					
K_m	0.029 $\pm$ 0.003	0.036 $\pm$ 0.006	56.2 $\pm$ 8.3	0.26 $\pm$ 0.03	0.43 $\pm$ 0.02[e,f]
V_{max} [d]	0.039 $\pm$ 0.008	0.0044 $\pm$ 0.0004	0.85 $\pm$ 0.15	0.068 $\pm$ 0.007	N/A
K_i values for inhibitors					
Berenil	54 $\pm$ 16	63 $\pm$ 3	>250	2.5 $\pm$ 0.8	2.4 $\pm$ 0.5[f]
Propamidine	3.7 $\pm$ 0.4	4.6 $\pm$ 0.7	>250	N.D.	1.9 $\pm$ 0.8
Stilbamidine	>250	56 $\pm$ 3	>250	N.D.	2.4 $\pm$ 0.3
Melarsen oxide	>100	>100	N.E., 100	0.63 $\pm$ 0.20	N.D.
Melarsoprol	N.D.	N.D.	N.D.	N.D.	0.54 $\pm$ 0.15[f]
Adenosine	N.E., 1000	N.E., 1000	N.E., 1000	0.80 $\pm$ 0.12	0.92 $\pm$ 0.06[e,f]
Adenine	N.E., 1000	N.E., 1000	N.E., 1000	0.42 $\pm$ 0.04	0.45 $\pm$ 0.04[f]

N.D., not determined; N.E., no effect at the indicated concentration.
[a] Using [^{3}H]pentamidine.
[b] Using [^{3}H]adenosine.
[c] K_i value for pentamidine.
[d] Expressed as pmol $(10^7$ cells$)^{-1}$ s^{-1}.
[e] K_m value.
[f] From De Koning and Jarvis (1999).

high-affinity pentamidine transport was therefore further investigated in procyclic trypanosomes.

PPT1 is a Proton Symporter. Although all the data on [^{3}H]pentamidine uptake in procyclics, and particularly the linear uptake over short and long intervals, is consistent with a one-transporter model for high-affinity pentamidine uptake, tests were devised to exclude the possibility of an uptake process driven by binding to an intracellular target. Uptake of 12.5 nM [^{3}H]pentamidine was determined at 22 and 0°C. Uptake at 22°C was linear for 100 s at a rate of 0.016 ± 0.003 pmol s^{-1}, after which all label had been taken up. At 0°C, the rate was reduced to 1.6% of the rate at room temperature ($2.5 \times 10^{-4} \pm 2.2 \times 10^{-5}$ pmol s^{-1}; eight time points spread over 10 min, $r^2 > 0.95$). The extent of the temperature sensitivity was indicative of a transporter-mediated mechanism of uptake rather than diffusion.

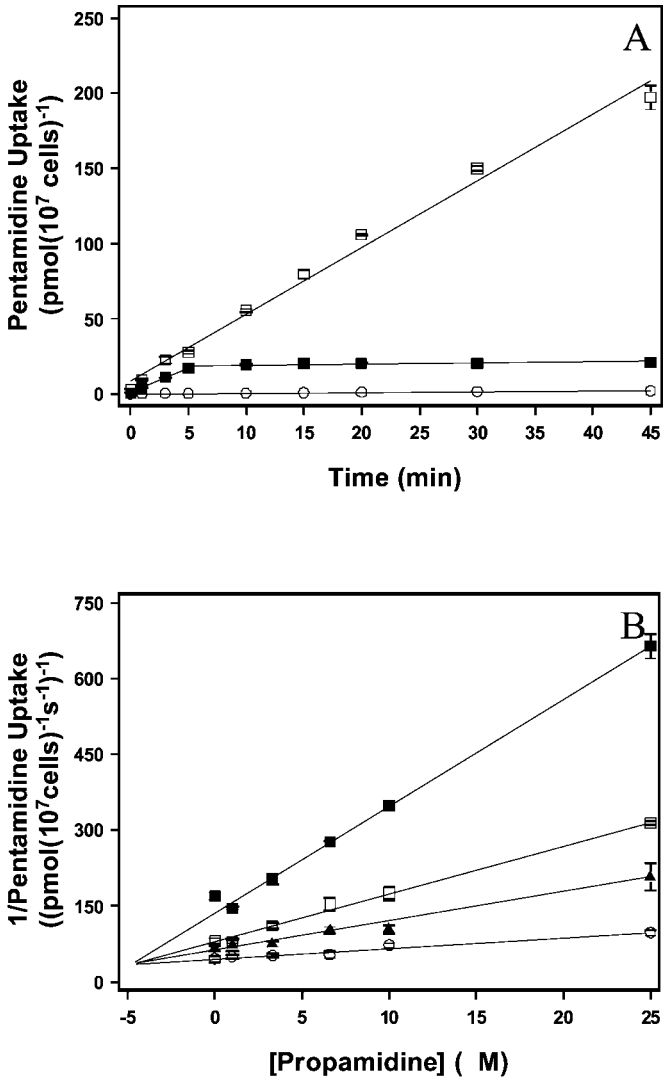

Fig. 4. Uptake of [^{3}H]pentamidine in *T. brucei brucei* procyclics. A, uptake of 50 nM [^{3}H]pentamidine in the presence (○) or absence (■) of 100 μM unlabeled pentamidine, or of 1 μM [^{3}H]pentamidine (□). After 5 min in 50 nM [^{3}H]pentamidine, virtually all permeant had been taken up by the parasites. B, Dixon plot of pentamidine uptake versus propamidine concentration. Pentamidine uptake in *T. brucei brucei* procyclics was determined as described under *Experimental Procedures*, at [^{3}H]pentamidine concentrations of 12.5 (■), 25 (□), 50 (▲), and 100 (○) nM, in the presence of various concentrations of propamidine. r^2 was >0.97 for all four lines, which intersected at x = −4.8.

In a further experiment, procyclic cells were permeabilized with 500 μg ml^{-1} of lysolecithin. K_m and V_{max} values in permeabilized and control cells were determined as described above. If these kinetic parameters represented binding constants and maximum rate of binding (limited by diffusion into the cell), K_m would be predicted to be unaltered and V_{max} increased in the permeabilized cells. However, it was observed that, although the K_m value was very similar to that of control cells (25 ± 5 versus 40 ± 1 nM, respectively), the V_{max} value was reduced by 67% in the lysolecithin-treated group. In the same experiment, [^{3}H]adenosine transport was found to be reduced by 73% after lysolecithin treatment. These results indicate that, like [^{3}H]adenosine, uptake of [^{3}H]pentamidine is concentrative, active transport. The reduced uptake rates following partial permeabilization may be the result of pentamidine efflux or of the collapse of plasma membrane ion gradients.

Many transport processes in protozoa (Zilberstein, 1993), including adenosine and hypoxanthine transport in procyclic *T. brucei brucei*, are examples of secondary active transport (De Koning and Jarvis, 1997a; De Koning et al., 1998), with the protonmotive force supplying the energy for concentrative uptake of nutrients. We therefore investigated whether the pentamidine transporters described here are likewise proton symporters. The uptake of pentamidine by PPT1 was independent of sodium, as replacement of Na^+ by *N*-methyl-D-glucamine in the transport buffer did not affect the rate of 100 nM [^{3}H]pentamidine uptake over a 5-min period (0.068 ± 0.005 and 0.072 ± 0.002 pmol $(10^7$ cells$)^{-1}$ s^{-1}, respectively; linear regression of eight points per line, $r^2 = 0.96$ and 0.99). However, PPT1-mediated pentamidine uptake was inhibited by the proton ionophore CCCP, with an IC_{50} value of 4.2 ± 0.3 μM (based on six points up to 40 μM). This value is very similar to values obtained for inhibition of the U1 and P1 adenosine transporters in *T. brucei brucei* uracil procyclics (3.8 ± 0.3 and 2.5 ± 0.4 μM, respectively), which are most probably proton symporters (De Koning and Jarvis, 1998; De Koning et al., 1998).

[^{3}H]Pentamidine transport was also inhibited by the Na^+/H^+ exchanger nigericin and the Na^+/K^+ exchanger gramicidin (Table 2), ionophores that acidify the *T. brucei brucei* cytoplasm or depolarize the plasma membrane, re-

TABLE 2

Effects of ionophores, NEM, and DCCD on PPT1-mediated pentamidine uptake

Procyclic trypanosomes were incubated with 100 nM [^{3}H]pentamidine as described under *Experimental Procedures*. Preincubation with inhibitors was 3 min. These data are the average ± S.E. of three independent expts., each performed in quadruplicate and using identical incubations with the same amount of solvent (1% EtOH) as a control.

Test Compound (μM)	Pentamidine Uptake (% of Control)	Protonmotive Force (mV)[a]
Control	100	−80.8 ± 2.2
CCCP (5)	56.4 ± 2.2	−36.0 ± 4.2
CCCP (10)	29.6 ± 0.6	−22.8 ± 3.7
Nigericin (5)	86.2 ± 1.2	−64.2 ± 4.9
Nigericin (10)	68.7 ± 2.0	−40.5 ± 4.8
Nigericin (20)	44.0 ± 0.8	−29.1 ± 4.8
Gramicidin (1)	18.0 ± 3.7	−20.1 ± 5.6
DCCD (10)	102 ± 5	−73.4 ± 3.2
DCCD (100)	30.8 ± 3.4	−19.8 ± 8.2
NEM (1000)	41.7 ± 0.69	−26.8 ± 3.6

[a] These values were obtained from De Koning et al. (1998).

spectively (De Koning et al., 1998). These results indicate that reduction of either intracellular pH or plasma membrane potential will inhibit [^{3}H]pentamidine uptake, consistent with PPT1-mediated transport being dependent on the protonmotive force. In support of this model, NEM and DCCD, sulfhydryl and carboxyl reactive agents, respectively, which have been shown to inhibit the *T. brucei brucei* plasma membrane proton pump (Thissen and Wang, 1991; Defrise-Quertain et al., 1996), also inhibited PPT1-mediated pentamidine uptake (Table 2). Plotting the effects of NEM, DCCD, and the ionophores on pentamidine uptake and on protonmotive force against each other produced a linear correlation (Fig. 5). Pentamidine uptake by HAPT1 in bloodstream forms was similarly found to be inhibited by CCCP, with 10 μM inhibiting >90% of 12.5 nM [^{3}H]pentamidine uptake in the presence of 250 μM adenosine.

Implications for Drug Resistance in African Trypanosomes. The presence of two pentamidine transporters in addition to P2 provides an explanation for why the arsenical-resistant *T. brucei brucei* clone RU15, developed from the same strain as used in the present study, but lacking detectable P2 transporter activity, was only very slightly cross-resistant to pentamidine (Fairlamb et al., 1992). The same clone (33-fold-resistant to melarsen oxide in vivo) was cross-resistant to stilbamidine and berenil (resistance factors of 38- and 32-fold, respectively), which are not transported by HAPT1 and LAPT1, and only moderately (6-fold) cross-resistant to propamidine, which may be taken up by HAPT1. It thus appears that the model of three distinct diamidine transporters presented here could form a basis to explain cross resistance patterns of drug resistance, at least in *T. brucei brucei*, and possibly other African trypanosomes. In agreement with such a model, clinical resistance to melarsoprol is a fast-growing problem, while resistance to pentamidine or suramin is reportedly all but absent (Kaminsky and Mäser, 2000). In laboratory strains with induced arsenical resistance, cross-resistance to berenil is also more widespread than to pentamidine (Bacchi, 1993). However, while this model may be relevant in many cases of drug resistance in African trypanosomes, it must be noted that not all observed drug resistance is necessarily due to changes in transporter function. The *T. brucei brucei* strain PR32.6 described by Berger et al. (1995) was resistant to pentamidine but not deficient in pentamidine accumulation or P2 transporter activity. It should also be noted that melarsoprol-pentamidine cross-resistance does occur in some laboratory strains (Kaminsky and Mäser, 2000). In most cases, however, the resistance had been induced by prolonged exposure to sublethal doses of melarsoprol, a treatment that is highly mutagenic, and may induce changes in additional transporters along with P2. A well documented example is the 6-fold reduction in P1-mediated adenosine transport in RU15 (Carter and Fairlamb, 1993; De Koning et al., 2000a).

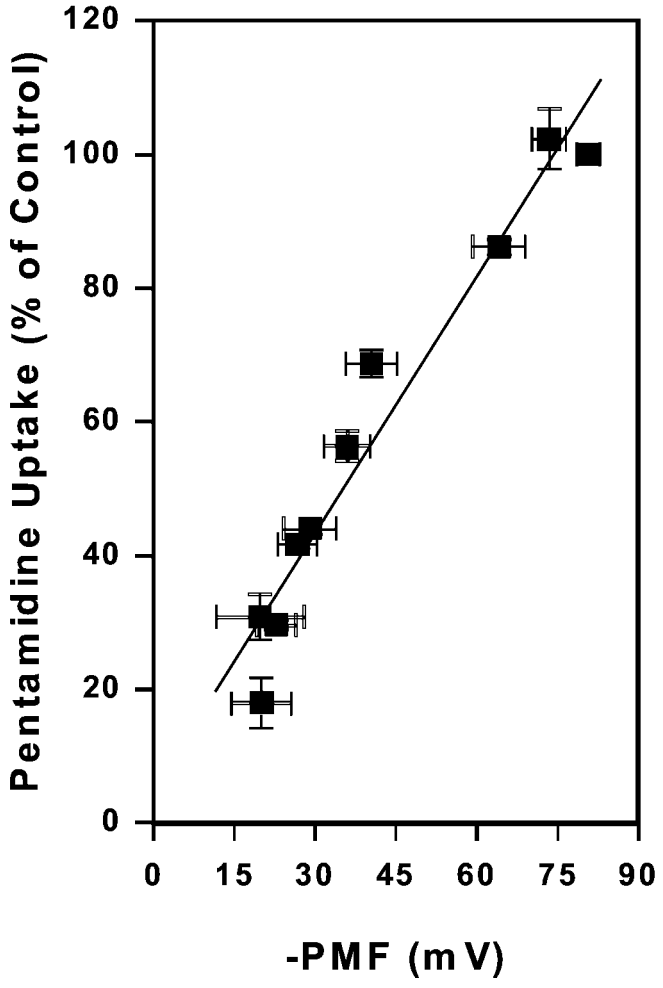

Fig. 5. Pentamidine uptake in *T. brucei brucei* procyclics is sensitive to ionophores and inhibitors of the plasma membrane proton pump. The effects of ionophores and other compounds on protonmotive force (PMF) and on 12.5 nM [^{3}H]pentamidine uptake in *T. brucei brucei* procyclics, as listed in Table 2, were plotted against each other to yield a straight line (linear regression, $r^2 = 0.95$).

In summary, the mechanism of uptake for a vital class of trypanocides, the diamidines, has been studied using [^{3}H]pentamidine. Two novel transporters are being reported here, and a model for drug transport in trypanosomes has been proposed. This model appears to adequately describe cross-resistance to trypanocides in laboratory strains and clinical isolates.

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