Uptake of Diamidine Drugs by the P2 Nucleoside Transporter in Melarsen-sensitive and -resistant Trypanosoma brucei brucei

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The African trypanosome, Trypanosoma brucei brucei, possesses at least two nucleoside transporter systems designated P1 and P2, the latter being implicated in the selective uptake of melaminophenyl arsenical drugs. Since arsenical-resistant trypanosomes show cross-resistance in vivo to aromatic diamidines, we have investigated whether these drugs are also substrates for the P2 nucleoside transporter. In melarsen-sensitive T. b. brucei, the diamidines, including the commonly used trypanocides, pentamidine and berenil, were found to abrogate lysis induced by the P2 transport of melarsen oxide in vitro. Measurement of [ring-^3H]pentamidine transport in melarsen-sensitive T. b. brucei, demonstrated that uptake is carrier-mediated, with a K_m of 0.84 μM and a V_max of 9.35 pmol s^-1 (10^9 cells)^-1. Pentamidine transport appears to be P2-mediated in these cells, as pentamidine strongly inhibited uptake of 2',5',8'-Hadenosine by the P2 transporter, with a K_i of 0.56 μM. Furthermore, [ring-^3H]pentamidine transport was blocked by a number of P2 transporter substrates and inhibitors, as well as by other diamidine drugs. Analysis of the uptake of pentamidine and other diamidines in melarsen-resistant trypanosomes in vitro and in vivo, which also show differential levels of resistance to these compounds in vivo, indicated that P2 transport was altered in these cells and that accumulation of these drugs was markedly reduced.

African trypanosomiasis continues to be a major public health and veterinary problem in many parts of Africa (1, 2). Treatment of the disease in humans and animals is confounded by the limited repertoire of drugs and by the emerging threat of drug resistance in the field (3, 4). One approach to the development of new drugs for the treatment of African sleeping sickness is the elucidation of the mode of action of existing drugs and the underlying mechanisms of drug resistance. The aromatic diamidine, pentamidine, is one of the most frequently administered drugs in the treatment of the early stage of the disease (5). However, despite more than 50 years of its use in the field, little is known about pentamidine's mode of action or the mechanisms which mediate pentamidine resistance (6, 7). Cross-resistance to pentamidine and other diamidines and to the melaminophenyl arsenicals has been observed frequently in laboratory strains of African trypanosomes (8–14), but how this resistance is conferred remains elusive.

Recently, we identified a novel P2 nucleoside transporter in T. b. brucei, which appears to mediate the uptake of trivalent melaminophenyl arsenical drugs (15). P2 transport was observed to be altered in a melarsen-resistant T. b. brucei clone, suggesting that resistance to these drugs may be conferred by a decreased accumulation of the drug due to alterations in P2 transport. The melarsen-resistant clone also displayed differential levels of resistance to various diamidines in vivo (10). In this report, we show that pentamidine and other diamidines are P2 transport substrates, and, furthermore, that the differential levels of resistance to the diamidine drugs displayed in vivo may be due to alterations in P2 transport.

EXPERIMENTAL PROCEDURES

Chemicals—[ring-^3H]Pentamidine (38.4 Ci mmol^-1) was custom-synthesized by Amersham International Plc (Little Chalfont, Buckinghamshire, UK) from dibromopentamidine as described previously (16). [2',5',8'-H]Adenosine (56 Ci mmol^-1) was also purchased from Amersham International Plc. Adenine, inosine, piperydine, and diminazene acetate (berenil) were all purchased from Sigma (Poole, Dorset, UK); adenosine from Boehringer Mannheim (Lewes, East Sussex, UK); dilazep from Tocris Cookson Chemicals (Langford, Bristol, UK); tetramethylammonium chloride and heptane sulfonate from Aldrich Chemical Co. (Gillingham, Dorset, UK). All other reagents were purchased at the highest purity available.

Both melarsen oxide and melarsoprol were generously provided by Spacila, Rhône-Poulenc (Paris, France), and the disulfonate salts of stilbamidine, hydroxystilbamidine, propamidine, dibromopentamidine, and pentamidine by Rhône-Poulenc (formerly May & Baker; Dagenham, Essex, UK).

Organisms—Two clones of T. b. brucei S427 were used in these studies: c118 (MITat 1.5) (17), which is susceptible to all melaminophenyl arsenicals and diamidines, and RU15, which was derived from c118 by sequential passage in mice in the presence of increasing concentrations of sodium melarsen (10). In mice, RU15 is highly cross-resistant to all melaminophenyl arsenicals and shows differential levels of resistance to diamidines (14). Both clones were amplified from stablates in Sprague-Dawley rats (300–500 g) and isolated by exsanguination. Trypanosomes were purified from blood by chromatography on DEAE-52 cellulose (Whatman, Maidstone, Kent, UK) (18) and maintained at 4 °C in CBSS, an RPMI-derived basal salt solution (10).

In Vitro Cell Lysis Assay—Cell lysis induced by melarsen oxide was measured at 37 °C in a thermostatted Beckman DU70 single-beam spectrophotometer by the associated decrease in absorbance at 750 nm.

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Transport of Aromatic Diamidines in African Trypanosomes

(10, 15). Trypanosomes (10^7) were prewarmed to 37 °C and added to 1-ml cuvettes containing CBSS (37 °C) plus 0.5 μM melarsen oxide and various concentrations of berenil, stilbamidine, propamidine, and pentamidine. Control samples containing neither drug, 0.5 μM melarsen oxide, or diamidine drugs alone were also included in each assay.

Uptake Studies—Linear rates of uptake for [3H]adenosine and [3H]pentamidine were measured either at 3 s intervals over 7 s for kinetic studies or at 5-s intervals over 30 s for inhibitor studies, as described (15, 19). At zero time, 0.1 ml of bloodstream trypanosomes (4 × 10^7 cells ml^-1), prewarmed to 25 °C, were pipetted sequentially into a series of Eppendorf tubes at 25 °C, containing 0.1 ml of silicone oil overlaid with 0.1 ml of CBSS supplemented with 1% (mass/volume) bovine serum albumin (BSA) containing either [3H]pentamidine or [3H]adenosine plus or minus other test compounds. Uptake was terminated by the rapid separation of cells from the radiolabeled layer by centrifugation in an Eppendorf 5414 microcentrifuge. The radiolabel layer was carefully removed by aspiration, and the oil layer was washed twice with ice-cold CBSS. The oil layer was then carefully removed without disturbing the cell pellet, and cells were extracted overnight at 4 °C with either 0.1 ml of 2% (v/v) TritonX-100 (scintillation grade from without disturbing the cell pellet, and cellswereextractedovernightat

twice with ice-cold CBSS. The oil layer was then carefully removed byaspiration, and the oil layer was washed
twice with ice-cold CBSS. The oil layer was then carefully removed without disturbing the cell pellet, and cells were extracted overnight at

of the treated rat plasma and DEAE column eluate supernatant were also analyzed by HPLC.

RESULTS

Effects of the Diamidines on Melarsen Oxide-induced Lysis of T. b. brucei— Within minutes of exposure to low concentrations of melarsen oxide or other melaminophenyl arsenicals, trypanosomes rapidly lose motility and lyse, losing their ability to scatter light. This property has been used to develop a spectrophotometric assay to identify compounds that might abrogate the lytic effect of melarsen oxide by competing for uptake on the P2-adenosine transport system (15). Fig. 1 shows the effect of pentamidine on this process against melarsen-sensitive cells. A 10-fold excess of pentamidine significantly delays cell lysis with complete abrogation occurring over the 30-min incubation with a 25–50-fold excess of pentamidine. Similar, but less pronounced dose-dependent protection against lysis was observed with other trypanocidal diamidines, including berenil, hydroxystilbamidine, stilbamidine, and propamidine (data not shown), suggesting that these compounds could also compete for uptake with melarsen oxide by the P2 transporter.

Transport Kinetics of [3H]Pentamidine in T. b. brucei—Linear rates of uptake for [3H]pentamidine into melarsen-sensitive T. b. brucei could be determined over 7 s at 25 °C in the 0.125–5 μM range. As shown in Fig. 2, uptake is saturable with an estimated K_m of 0.84 ± 0.16 μM and a V_max of 9.35 ± 0.58 pmol s^-1 (10^6 cells)^-1. To determine whether [3H]pentamidine uptake is mediated by the P2 nucleoside transporter in T. b. brucei, the inhibition of P2 [3H]adenosine transport (1 and 10 μM) by pentamidine (0.25–1.5 μM) was investigated at 25 °C in the presence of 1 mM inosine to saturate the P1 adenosine transporter. The results, presented as a Dixon plot in Fig. 3 (22), indicate that pentamidine is a potent inhibitor of P2 adenosine transport with a K_i of 0.48 μM. Similar results were obtained in three additional experiments yielding a mean K_i of 0.56 ± 0.08 μM, n = 4. However, in the converse experiment, which measures the inhibition of [3H]pentamidine transport (1, 5, and 10 μM) by adenosine (0.5–5 μM), we were unable to determine a K_i value for adenosine. This finding raised the possibility that pentamidine is not a substrate for the P2 transporter, but merely an inhibitor. To investigate this possibility, the effects of a 10-fold excess of a number of other P2-transported substrates and inhibitors were assessed on the initial
Pentamidine Transport into Melarsen-resistant Trypanosomes—Previously, we have reported that melarsen-resistant (cRU15) trypanosomes have altered transport kinetics for adenosine in vitro, such that uptake of [3H]adenosine on the P2 transport system cannot be measured over brief intervals (15). To examine whether P2 pentamidine transport is also altered in this clone, P2 transport kinetics for [3H]pentamidine were measured under the same conditions given in Fig. 2 for melarsen-sensitive (c118) trypanosomes. As previously reported for adenosine (15), uptake of pentamidine on the P2 transporter could not be detected in cRU15 over a 1- to 7-s time course (results not shown). However, pentamidine is still effective against cRU15 infections in mice with an ED50 value of 0.93 mg kg−1, 1.6-fold higher than that observed for c118 of 0.59 mg kg−1 (14). Moreover, the other aromatic diamidines are also active in vivo against cRU15, albeit at higher doses than those required for c118 (see Table I). These findings could imply that these drugs are still accumulated but that P2 transport kinetics are markedly diminished in cRU15. To investigate this possibility, the uptake of 0.5 μM and 10 μM [3H]pentamidine was measured over a 3-h time course in both c118 and cRU15 (Fig. 4, a and b). Pentamidine accumulates rapidly in c118 achieving an intracellular concentration of 0.95–1.00 nmol (108 cells)−1 within the first 30 min (Fig. 4a, open circles). Uptake ceases at this time, as essentially all of the available drug is depleted from the medium. Based upon an intracellular volume of 5.8 μl (108 cells)−1 (23), this amount of drug is equivalent to an intracellular concentration of 0.17 mM and represents an accumulation of >1000-fold over the final external concentration. In contrast, cRU15 accumulates 0.5 μM [3H]pentamidine to only 0.125 nmol (108 cells)−1 (0.021 mM), which is 8-fold lower than for c118 (Fig. 4a, closed circles). This difference between cRU15 and c118 was also observed in cells after exposure to 10 μM [3H]pentamidine (Fig. 4b), which again revealed differences in the intracellular level of drug (1.2 mM for c118 versus 0.12 mM for cRU15 after 3 h).

The initial rates of uptake of pentamidine by cRU15 can be estimated from Fig. 4 to be 0.069 and 0.370 pmol s−1 (108 cells)−1 for 0.5 μM and 10 μM pentamidine, respectively. Although initial rates cannot be accurately determined for c118 from Fig. 4, they can be calculated using the Michaelis-Menten equation to be 3.49 and 8.63 pmol s−1 (108 cells)−1, respectively. Thus, cRU15 appears to take up pentamidine at an initial rate that is 20- to 50-fold slower than c118.

HPLC Analysis of In Vitro Uptake—In order to study the uptake of the other unlabeled diamidine drugs, cellular diamidine content was measured using an HPLC system originally
designed for pentamidine and its metabolites (21). An initial experiment established that uptake of 1 μM pentamidine was essentially linear with no loss of motility over a 3-h incubation in RPMI/BSA at 37 °C (data not shown). After 3 h, the cells contained 6.2 nmol (10^8 cells)^{-1} which represents an intracellular concentration of 1.1 mM. Since this represents 62% of the total pentamidine added, a final concentration gradient of 2,800-fold between cells and medium can be calculated. In subsequent experiments, paired samples of c118 and RU15 were incubated with either 1 μM pentamidine, stilbamidine, or propamidine for 3 h, in order to compare accumulation of the compounds. In each case, melarsen-resistant trypanosomes (cRU15) took up 3–6-fold less diamidine than the wild-type (Table II). In both cell types, pentamidine achieved a higher intracellular concentration than propamidine or stilbamidine. However, there was no obvious correlation between the ratio of amount of drug taken up in vitro and the resistance factors previously observed in vivo in mice (10, 14).

HPLC Analysis of in Vivo Uptake—For these experiments, heavily infected rats were treated with 4 mg kg^{-1} of either pentamidine, stilbamidine, or propamidine and trypanosome plasma samples were isolated 4 h post-drug administration. Note that a 4-h time point was chosen because no significant reduction in parasitemia occurs during this time (data not shown). At 4 h, the plasma concentration of each diamidine was below the limits of detection of the HPLC method, indicating that the drug had largely been cleared from the bloodstream. There was no significant loss of drug into either the DEAE-column buffer or the buffer used to resuspend the parasites indicating that pentamidine is not lost from the cells during isolation and purification (data not shown). HPLC analysis of each trypanosomal cell extract (Table III) shows that in vivo c118 also accumulates pentamidine to higher intracellular levels than either stilbamidine or propamidine. Moreover, cRU15 accumulates less of each diamidine. However, unlike in vitro accumulation studies, each diamidine was concentrated by cRU15 to approximately the same intracellular concentration (0.11–0.16 nmol (10^8 cells)^{-1}), indicating that the intracellular accumulation of drug may also be dependent on drug pharmacokinetics.

**DISCUSSION**

Our previous work suggested that uptake of the melamino-phenyl arsenical drugs into T. b. brucei is mediated by a P2 nucleoside transporter (15). The present study demonstrates that uptake of pentamidine is also carrier-mediated with several lines of evidence suggesting that this involves the same P2 transporter. First, uptake of pentamidine exhibits saturation kinetics consistent with a carrier-mediated mechanism. The kinetic parameters determined here for T. b. brucei (K_m = 0.84 μM and V_max = 9.35 pmol s^{-1} (10^8 cells)^{-1} at 25 °C) are broadly in agreement with previous estimates for T. b. brucei EATRO 110 (K_m = 2.7 μM; V_max = 6.1 pmol s^{-1} (10^8 cells)^{-1} at 37 °C) (24). Second, pentamidine is a potent inhibitor of adenosine uptake on the P2 transporter with a K_i (0.56 μM) similar to its K_m (0.84 μM). Although these data could be compatible with simple competitive inhibition, where pentamidine competes with adenosine for uptake on the P2 carrier and vice versa, the converse experiment did not yield the predicted reciprocal kinetic parameters: adenosine only inhibits pentamidine transport at concentrations >10 μM indicating that the K_i must be significantly greater than its K_m (0.59 μM) for the P2 transporter (15). The reason for this discrepancy is not known. Third, with this notable exception, all other inhibitors of arsenical-induced lysis, which are also inhibitors (or competitive substrates) of adenosine uptake on the P2 transporter (15), strongly inhibit transport of pentamidine. In the case of the diamidines, these findings are consistent with a previous report that propamidine, stilbamidine, and hydroxystilbamidine are competitive inhibitors of pentamidine transport with K_i values of the same order as the K_m determined for pentamidine (24). Fourth, melarsen-resistant cells exhibit profound alterations in their ability to transport both adenosine (15) and pentamidine (this study) via the P2 system in the short term (seconds), which is reflected in their decreased ability to accumulate both pentamidine and other diamidines in the longer term (minutes or hours).

Our current results show that pentamidine can be rapidly accumulated and concentrated by several orders of magnitude within the cell. Others have shown that pentamidine transport can be partially inhibited by a number of metabolic inhibitors, suggesting involvement of an energy-dependent process (24). However, it would be premature to discard the possibility that the high intracellular levels observed may be due to binding to intracellular sites, such as kinetoplast DNA (25, 26). HPLC analysis of trypanosomal extracts reported here and elsewhere...
(16) indicates that there is no significant metabolism of diamidine drugs in the cell; thus, the maintenance of a concentration gradient by metabolic conversion of these drugs is unlikely.

Loss or alteration in the kinetic properties of the P2 transporter offers an attractive explanation for the well-documented nonreciprocal cross-resistance between the melaminophenyl arsenicals and diamidine classes of trypanocidal drugs (8–14). Although the intracellular targets for these drugs are not known with any certainty, it is likely that they are different. Thus, the frequently observed nonreciprocal nature of this cross-resistance could depend on the relative contributions made either by alterations in the intracellular targets or by alterations in the P2 transporter to the overall resistance of the cell. However, this hypothesis does not account entirely for the differential levels of resistance of trivalent melaminophenyl arsenicals and diamidines in our melarsen-resistant line (14). Possibly, the wide range of resistance factors observed could depend on the extent to which these drugs can be accumulated to achieve lethal intracellular concentrations in cRU15, which, in turn, would depend on their pharmacokinetic profile. Precise information on the plasma pharmacokinetics of the biologically active forms of many of these drugs is lacking in rodents. However, prophylactic activity could provide some indication of gross pharmacological properties. Significantly, none of the melaminophenyl arsenicals have any prophylactic activity in rodents when subsequently challenged with trypanosomes (7), correlating with cRU15 showing moderate to high resistance against these compounds (26–69, and 121-fold for melarsen oxide, melarsoprol, and trimelarsen, respectively) (14). In contrast, with the notable exception of berenil (27), all of the diamidines possess considerable prophylactic activity (7, 28) with cRU15 showing low resistance to pentamidine, stilbamidine, and propamidine (1, 5, 7, and 7.7-fold, respectively) versus moderate resistance to berenil (24-fold) (14). Although comparative studies failed to distinguish between the prophylactic activity of pentamidine and stilbamidine (29), radiotracer studies in mice suggest that stilbamidine is excreted more rapidly than pentamidine (50% elimination in 2 and 5–6 days, respectively) (30–32). Unfortunately, most of the drug that is retained in the body is sequestered in tissues, especially liver and kidney, and the residual amounts in plasma were too low to measure. Nonetheless, these data support a correlation between pharmacological properties and resistance for these drugs. Our failure to find any striking difference between the intracellular concentrations of stilbamidine and pentamidine in cRU15 following 4-h exposure in vivo does not necessarily invalidate this hypothesis, since this interval may not be sufficient to reveal marked differences in diamidine concentrations necessary to achieve cell killing. It is not possible to extend this time course much beyond 4 h since pentamidine clears the parasitemia shortly thereafter (16). Further work is required to test this hypothesis.

In conclusion, it appears that, like the melaminophenyl arsenicals, the diamidines are also accumulated in T. b. brucei by a P2 nucleoside transporter and that resistance to the diamidine drugs may be partly conferred by alterations in P2 transport and the pharmacokinetic properties of the drug. The precise mechanism by which P2 transport is altered is unclear at present, but it may be envisaged that it arises from either point mutations within the gene thereby altering $K_m$, $V_{max}$, or both of the transporter, or, alternatively, the expression of the P2 transporter may be down-regulated. Elucidation of the precise mechanism of P2-mediated resistance awaits study by genetic analysis.

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