Changes in adenosine transport associated with melaminophenyl arsenical (Mel CY) resistance in *Trypanosoma evansi*: down-regulation and affinity changes of the P2 transporter

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(*Received 25 February 2003; revised 8 July 2003; accepted 8 July 2003*)

SUMMARY

Studies of the kinetics of adenosine transport were carried out on the P1 and P2 transporters of drug-sensitive *Trypanosoma evansi* and its cloned derivative, resistant to the melaminophenyl arsenical Mel CY. Characterization of adenosine uptake was made by estimation of the maximum concentration taken up at time infinity (A_{max}). A_{max} on the P2 transporter of sensitive *T. evansi* was greater than A_{max} on the P1 transporter. A_{max} of the P2 transporter was significantly decreased in drug-resistant trypanosomes. The effect of adenosine concentration and inhibitors, on the rate of adenosine uptake, was described by Michaelis-Menten equations. In sensitive *T. evansi*, the maximum velocity of adenosine uptake (V_{max}) of the P2 transporter was 2-fold greater than V_{max} of the P1 transporter. The V_{max} of the P2 transporter in resistant parasites was reduced 9-fold. The binding constants K_m and K_i on the P2 transporter of resistant *T. evansi*, showed that resistance was associated with an increased affinity for adenosine, and a decreased affinity for adenine and Berenil. We suggest that resistance to melaminophenyl arsenicals in *T. evansi*, occurs via 2 mechanisms: (1) a reduction in the number of expressed P2 transporter molecules resulting in decreased uptake of melaminophenyl arsenicals; (2) a change in the binding properties of the P2 transporter.

Key words: P2 transporters, down-regulation, affinity changes, *Trypanosoma evansi*.

INTRODUCTION

Recent studies on purine transport in trypanosomes have identified at least 2 different nucleoside transporter systems in drug-sensitive trypanosomes (Carter & Fairlamb, 1993; Carter, Berger & Fairlamb, 1995; Barrett *et al*. 1995; Ross & Barns, 1996; Scott, Tait & Turner, 1997; Suswam *et al*. 2001). The P1 transporter system mediates uptake of adenosine, inosine and guanosine, and has been shown to be present in the bloodstream forms as well as the procyclic stages of *T. brucei* (Sanchez *et al*. 2002). The P2 transporter system mediates uptake of adenosine, adenine and several anti-trypanocidal drugs, including the melaminophenyl arsenicals (Carter & Fairlamb, 1993; de Koning, Watson & Jarvis, 1998) and has been detected only in the bloodstream forms (Sanchez *et al*. 2002). In addition, 4 nucleobase transport activities have also been identified. The H1, H2, H3 mediate transport of hypoxanthine, guanine and adenine (de Koning & Jarvis, 1997). H1 is present in the procyclics, and H2 and H3 is present in both bloodstream forms and procyclic stages. A U1 transporter mediates transport of uracil in procyclic *T. brucei* (de Koning & Jarvis, 1998).

Increased interest in purine transport activities in trypanosomatids led to the cloning and characterization of the TbAT1, a P2-type nucleoside transporter (Maser *et al*. 1999) and the nucleoside transporter-2 gene, TbNT2 (which encodes a P1-type transporter) (Sanchez *et al*. 1999) in *T. brucei*. Sanchez and co-workers (Sanchez *et al*. 2002) later showed that the TbNT2 is indeed a member of a multigene family encoding 6 similar transporters with distinct biochemical properties (TbNT2/927 to TbNT7). These authors demonstrated that these transporters are differentially regulated in the life-cycle stages of *T. brucei*, and mediate uptake of purine nucleosides and in some cases the nucleobase, hypoxanthine.

These complexities in purine transport in trypanosomes highlight the need for more detailed biochemical characterizations of purine transporters in order to provide useful information for improved drug delivery as well as a better understanding of drug resistance phenotypes associated with purine transporters.

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Parasitology (2003), **127**, 543–549.
DOI: 10.1017/S003118200300413X Printed in the United Kingdom

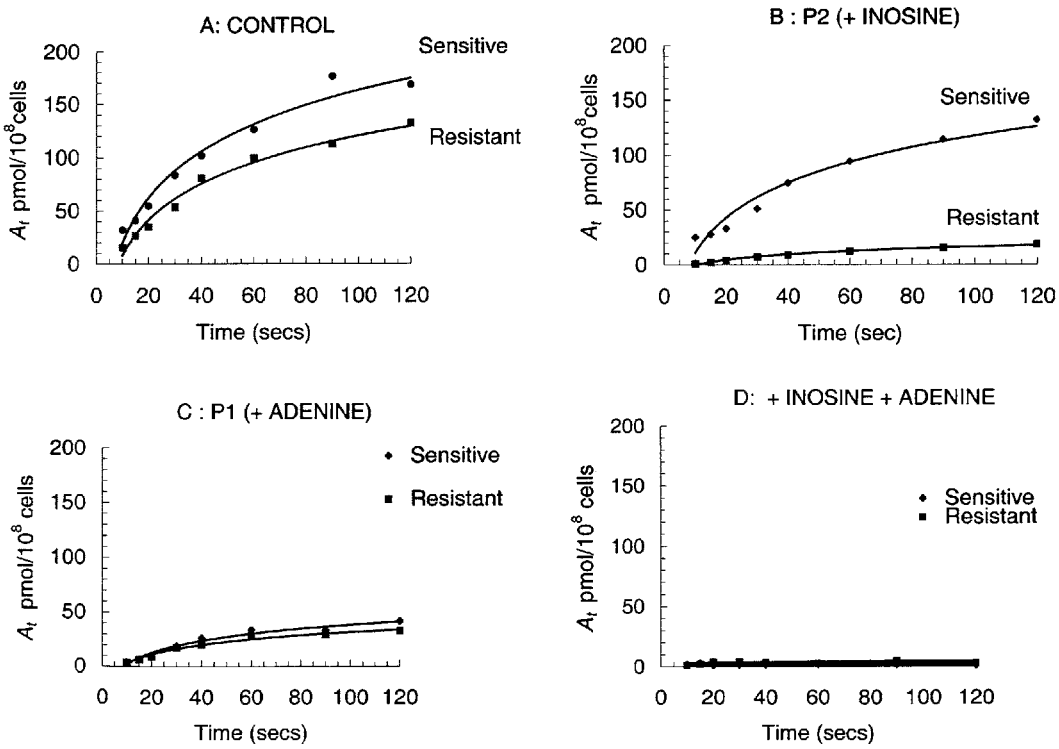

Fig. 1. Representative experiments showing adenosine uptake in drug-sensitive and drug-resistant *Trypanosoma evansi*. 2×10^7 trypanosomes were incubated with 5 μM tritiated adenosine either in the absence of inhibitor (A); or presence of 1 mM inosine, the P2 transporter (B), 1 mM adenine, the P1 transporter (C), or 1 mM of both adenine and inosine (D); at 37 °C. Incorporated radioactivity was measured at time-points between 10 and 120 sec. Trypanosomes were separated from transport medium by filtration and centrifugation through 0·45 μM cellulose filter. Transport was calculated from counts of decompositions of radioactivity recorded from a scintillation counter; and expressed as ρmoles/10^8 cells. Data were fitted to exponential plots using non-linear regression analysis. Note the non-zero start position. From these fittings transport rates were estimated as described in the text. Each experiment was repeated 4 times and similar results were obtained.

Carter & Fairlamb (1993) earlier showed that a loss or modification of P2 transporter is associated with resistance to the melaminophenyl arsenical group of trypanocides. Our studies using the monomorphic species, *T. evansi*, have shown that the P2 transporter was retained but the activity of the P2 was greatly decreased in several lines of arsenical-resistant *T. evansi* selected either *in vivo* or *in vitro* (Suswam *et al*. 2001). We show in this paper that the changes associated with resistance are a reduction in numbers of the transporter and modification of the affinity of the P2 transporter.

MATERIALS AND METHODS

Trypanosomes

We used a mouse-maintained drug-sensitive *T. evansi* clone (TREU 1840), and its *in vivo* derived Mel CY drug-resistant clone (CR3.1).

Adenosine uptake after addition of 5 μM adenosine

Uptake of adenosine over time was observed for 120 sec after immersion in 5 μM tritiated adenosine, in drug-sensitive and drug-resistant trypanosomes. Uptake on the P1 transporter was determined while the P2 transporter was inhibited with 1 mM adenine; uptake on the P2 transporter was measured while the P1 transporter was inhibited with 1 mM inosine. The total uptake over time by the P1 and P2 transporters together was also measured in the absence of inhibitors.

Trypanosomes, at a density of $2{\cdot}5 \times 10^8$ cells/ml, were separated from mouse blood, washed and re-suspended in transport buffer consisting of 25 mM HEPES, 120 mM NaCl, 5·4 mM KCl, 0·55 mM $CaCl_2$, 0·4 mM $MgSO_4$, 5·6 mM Na_2HPO_4, 11·1 mM glucose, pH 7·4; and containing 1% bovine serum albumin (Fairlamb *et al*. 1992). Two $\times 10^7$ pre-warmed cells were incubated at 37 °C in a total volume of 100 μl, in the presence or absence of inhibitors. At the start of the assay tritiated adenosine ([$^{2\text{-}3}$H], specific activity 185 Bq/mmol or 12·5 mCi/mmol) was added to produce a concentration of 5 μM for the experiments, and the reaction was stopped at different times by the addition of 100 μl of 5 mM ice-cold non-radio-isotope labelled adenosine in PBS. The reaction mixture was immediately filtered by centrifugation through a 0·45 μM acetate filter (SPIN-X, COSTAR), at 1000 ***g*** for 40 sec, and the filters were rinsed once with 0·5 ml of ice-cold adenosine solution

https://doi.org/10.1017/S003118200300413X Published online by Cambridge University Press

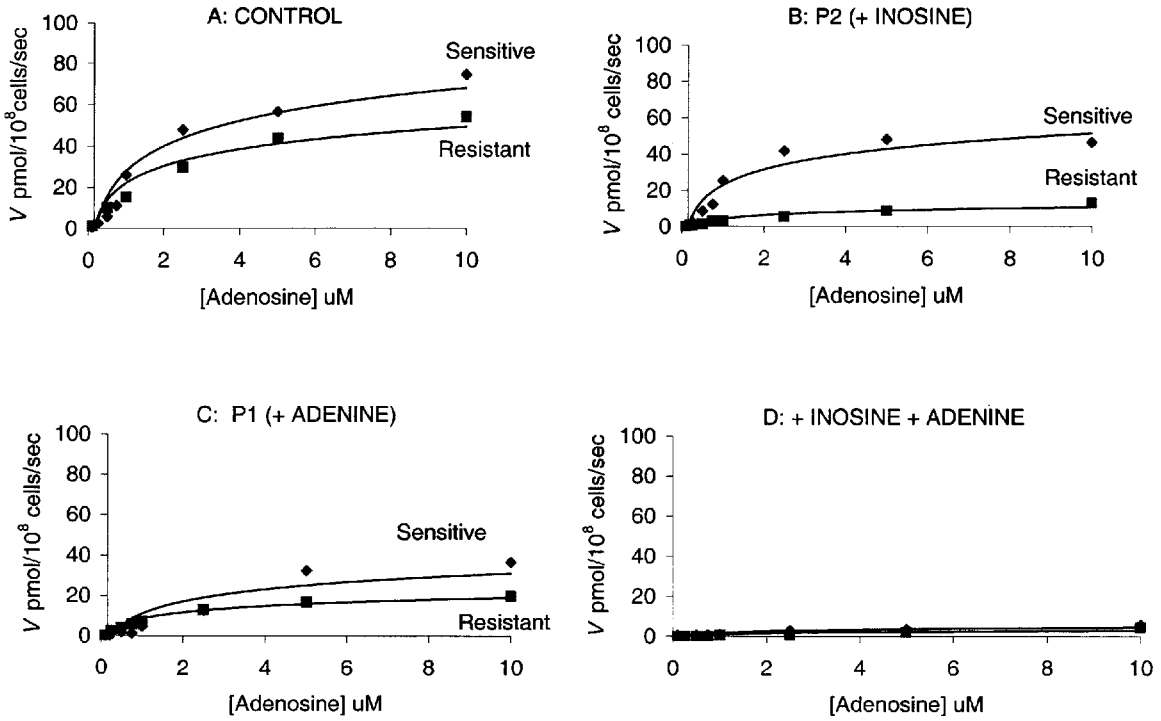

Fig. 2. Uptake of adenosine in drug-sensitive and drug-resistant *Trypanosoma evansi*: Effect of substrate concentration on uptake. 10^7 trypanosomes were incubated at 37 °C with varying concentrations of tritiated adenosine, in the absence of inhibitor (A); or presence of 1 mM inosine (B), 1 mM adenine (C), or 1 mM of both adenine and inosine (D). Uptake, V, was calculated from incorporated radioactivity and expressed as pmoles/10^8 cells. Data were fitted to rectangular hyperbolic plots and analysed using a non-linear regression analysis programme. Kinetic parameters were determined from these plots using Michaelis-Menten equations. Each experiment was repeated 3 times and similar results obtained.

at 1000 ***g*** for 1 min. The filters were dried overnight at 50 °C. Incorporated radioactivity was counted in 1 ml of scintillation cocktail (Packard, UK) using a Scintillation Counter. Background readings of radioactivity were determined by incubation of transport buffer and tritiated adenosine with no trypanosomes. Reactions were carried out in triplicate and experiments were repeated 3–4 separate times. Transport of adenosine was calculated from counts of decompositions of incorporated radioactivity, and expressed as ρmoles/10^8 cells.

The uptake of adenosine by our trypanosomes was not linear over the 120 sec period of the experiment, (Fig. 1) and showed an exponential increase over time. Our observations differed from other authors (e.g. de Koning & Jarvis, 1999) where the early uptake appeared linear. Since we observed an exponential uptake rate we used Equation 1 (a one-compartment open model with constant infusion and a single rate of adenosine removal from within the trypanosome, e.g. Gibaldi (1991)). The equation allows for metabolism and efflux of adenosine and describes the adenosine concentration uptake–time relationship:

$$A_t = t_0 + A_{max}(1 - e^{-t/T}), \quad (1)$$

where A_t = adenosine concentration (ρmoles/10^8 cells) at time, t (seconds); T = metabolism and efflux time-constant (seconds), it is equal to the reciprocal of the elimination rate; A_{max} = maximum adenosine concentration at time infinity (ρmoles/10^8 cells); t_0 is the correction (5–10 sec) for a non-zero start-time.

Effects of bath concentration of adenosine on the rate of uptake of adenosine

Trypanosomes separated from mice blood were washed and suspended in transport buffer at a density of $1{\cdot}25 \times 10^8$ trypanosomes/ml. Uptake of adenosine on the P1, P2, or both P1 and P2 transporters was measured in 1×10^7 trypanosomes essentially as described above, with few modifications. Tritiated adenosine was included at concentrations of 0·1, 0·25, 0·5, 0·75, 1·0, 2·5, 5·0, or 10·0 μM. Reactions were carried out in duplicate. The total uptake of adenosine was calculated from counts of decompositions of incorporated radioactivity, and expressed as ρmoles/10^8 cells.

Control experiments were performed in the absence of trypanosomes to correct for non-specific binding of radioactivity. The data were corrected for this value in each case. Each experiment was repeated 3 or 4 separate times.

The initial uptake rates ($V = A_{max}/T$ from Equation 1) were determined at different concentrations of adenosine in the bath and fitted to a modified form of the Michaelis-Menten:

$$V = (V_{max}[A]/([A] + K_m) + K_L[A]), \quad (2)$$

https://doi.org/10.1017/S003118200300413X Published online by Cambridge University Press

Table 1. A_{max} of adenosine transport on the P1 and P2 transporters in *Trypanosoma evansi*: transport rates

Adenosine transport	Drug sensitive (WT)	Drug resistant (CR3.1)
	A_{max} (ρmol/10^8 cells)	
Total uptake	140·6 ± 24·0	122·6 ± 13·0
Adenine present (P1)	67·7 ± 9·7	43·9 ± 2·0
Inosine present (P2)	112·5 ± 17·8	33·6 ± 8·5

where V = initial rate of uptake (ρmoles/10^8 cells/sec); V_{max} = maximum rate of uptake (ρmoles/10^8 cells/sec); $[A]$ = bath concentration of adenosine; K_m = concentration of adenosine, yielding 50% V_{max} and K_L, is the linear uptake (passive diffusion). The data obtained from each experiment by this method were corrected for the passive diffusion.

Each experiment was repeated 3 or 4 times and the data of single experiments fitted to determine the means ± S.E. of the constants of Equation 2.

Inhibition of adenosine uptake at the P1 and P2 transporters

The inhibition of adenosine uptake by inosine, adenine and Berenil® was studied in both drug-sensitive and drug-resistant trypanosomes. The effect of concentration of the inhibitors, inosine, adenine and Berenil® on adenosine uptake on the P1 transporters or P2 transporters was determined by measuring the uptake of tritiated adenosine by 10^7 trypanosomes in the presence of increasing concentrations of each inhibitor. The concentrations used were 0·01, 0·1, 1·0, 10, 50, 100, 1000 μM inosine; 0·01, 0·1, 1·0, 10, 50, 100 μM adenine; and, 0·01, 0·1, 1·0, 10, 50, 100, 500 μM Berenil®. The inhibition data were fitted to Equation 3:

$$V = V_{max}[S]/([S] + K_m(1 + I/K_i)), \quad (3)$$

where, V = initial velocity of uptake of adenosine (ρmoles/10^8 cells/sec); V_{max} = maximum initial velocity of uptake of adenosine (ρmoles/10^8 cells/sec); $[S]$ = concentration of substrate (adenosine, μM); K_m = dissociation constant (μM) for adenosine at the P1 or P2 transporter; I = concentration of inhibitor (μM); K_i = dissociation constant for inhibitor (μM). K_m was fixed as 0·07 μM for P1 and 0·74 μM for P2, respectively, in drug-sensitive trypanosomes; and 0·06 μM for P1, 0·1 μM for P2 in drug-resistant trypanosomes. These values were determined from the earlier rate of uptake studies.

Fitting and statistics

Equations 1, 2, and 3 were fitted to the data using non-linear regression (Microcal Origin). Means ± S.E. of the estimated parameters were calculated from 3 or 4 separate experiments and the data were analysed using an unpaired t-test.

RESULTS

Adenosine uptake, A_{max} *by the P1 and P2 transporters*

Fig. 1A shows a representative experiment in which the time-course of the total uptake of adenosine via the combined P1 and P2 transporter systems in drug-sensitive and drug-resistant trypanosomes was measured. The uptake was non-linear and had a non-zero start time (5–10 sec) that was described by Equation 1. Fig. 1B shows representative experiments of the uptake via the P2 transporter systems with the P1 transporters blocked with 1 mM inosine. Fig. 1C shows a representative experiment of the uptake via the P1 transporter system with the P2 transporters blocked with adenine. The overall uptake of adenosine in the drug-sensitive parasites appeared to be greater than that of the drug-resistant trypanosomes. Comparison of Fig. 1B (P2 transporters) and Fig. 1C (P1 transporters) shows that the P2 transporter of the drug-sensitive *T. evansi* has a bigger capacity compared to P1, and that the P2 capacity was significantly reduced in the drug-resistant clone. There was little or no transport of adenosine in the presence of saturating concentrations of both adenine and inosine, in either the drug-sensitive or drug-resistant *T. evansi* (Fig. 1D).

The mean ± S.E. values for the maximum concentration at time infinity (A_{max}), were estimated for the P1 and P2 transporters of the drug-sensitive trypanosomes from 4 similar experiments (Table 1). A_{max} of the P1 transporter in these parasites, was 67·7 ± 9·7 ρmol/10^8 cells and that is significantly ($P < 0·01$) less than the P2 transporter 112·5 ± 17·8 ρmol/10^8 cells and has 60% of its efficacy.

When we looked at the A_{max}, of the P2 transporter in the drug-resistant parasites (Fig. 1 and Table 1). It turned out that the A_{max}, 33·6 ± 8·5 ρmol/10^8 cells, was significantly smaller ($P = 0·016$) than A_{max} for drug-sensitive parasites and this represents only 30% of the P2 uptake capacity in drug-sensitive trypanosomes. Changes in the drug-resistant clone values (Table 1), for A_{max} of the P1 transporter did not reach statistical significance when the drug-sensitive trypanosomes and drug-resistant results were compared.

Effect of adenosine concentration on the rate of its uptake

The plots representing the effect of concentration of adenosine on the initial rate of uptake, V, in the drug-sensitive *T. evansi* are shown in Fig. 2. A summary of the kinetic parameters, V_{max} and K_m,

https://doi.org/10.1017/S003118200300413X Published online by Cambridge University Press

Table 2. Effect of substrate concentration on adenosine uptake, and kinetics of inhibition of adenosine uptake in *Trypanosoma evansi*: Michaelis-Menten kinetics

	Drug sensitive (WT)					Drug resistant (CR3.1)				
Transporter system	V_{max}	K_m of adenosine	K_i of inosine	K_i of adenine	K_i of Berenil	V_{max}	K_m of adenosine	K_i of inosine	K_i of adenine	K_i of Berenil
Adenine present (P1 transporter)	12·4 ± 2·3	0·07 ± 0·03	0·03 ± 0·004	N.A.	N.A.	6·7 ± 1·80	0·06 ± 0·02	0·063 ± 0·03	N.A.	N.A.
Inosine present (P2 transporter)	28·9 ± 5·10	0·74 ± 0·30	N.A.	0·3 ± 0·10	0·01 ± 0·01	3·3 ± 1·10	0·1 ± 0·02	N.A.	1·940 ± 0·30	Very high $>10\,\mu$M

N.A., Not applicable.
* V_{max} units = (pmol/10^8 cells/sec); K_m = (μM); K_i = (μM).

estimated from plots like those of Fig. 2B and C is given in Table 2. Table 2 shows that the P1 transporter saturates at a 10-fold lower concentration of adenosine with a K_m of $0{\cdot}07 \pm 0{\cdot}03\,\mu$M compared to the P2 transporter K_m of $0{\cdot}74 \pm 0{\cdot}3\,\mu$M; this is significantly different, $P < 0{\cdot}01$. The maximum rate of uptake, V_{max}, on the P2 transporter ($28{\cdot}9 \pm 5{\cdot}1$ pmol/10^8 cell/sec) was double that of the P1 transporter ($12{\cdot}4 \pm 2{\cdot}3$ pmol/10^8 cell/sec). The effect of substrate concentration on adenosine uptake in the drug-resistant *T. evansi* is also illustrated in Fig. 2 and a summary of the estimated parameters shown in Table 2. V_{max} and K_m values of the P1 transporter did not change significantly when compared to the drug-sensitive parasites. In contrast, P2 in the drug-resistant clone was significantly changed with V_{max} ($3{\cdot}3 \pm 1{\cdot}1$ pmol/10^8 cells/sec) reduced 9-fold ($P < 0{\cdot}05$) and the K_m ($0{\cdot}1 \pm 0{\cdot}02\,\mu$M) reduced 7-fold ($P < 0{\cdot}05$). So the P2 transport rate was reduced and the affinity for adenosine was increased with resistance.

Kinetics of inhibition of adenosine uptake in T. evansi

K_i values were determined for inhibition by inosine, adenine, and Berenil on the P1 or P2 transporters (Table 2). The K_i for inosine on the P1 transporter was $0{\cdot}03 \pm 0{\cdot}004\,\mu$M; the K_i for adenine on the P2 transporter was $0{\cdot}3 \pm 0{\cdot}1\,\mu$M; and the K_i for Berenil on the P2 transporter was $0{\cdot}01 \pm 0{\cdot}01\,\mu$M. There appeared to be a moderate increase in the mean estimate for inosine K_i on the P1 transporter for drug-resistant parasites ($0{\cdot}063 \pm 0{\cdot}03\,\mu$M), but this did not reach significance. The increase in adenine K_i on the P2 transporter was much bigger, 7-fold, to $1{\cdot}94 \pm 0{\cdot}3\,\mu$M, and was statistically significant ($P = 0{\cdot}03$). We were not able to detect significant inhibition by Berenil on the P2 transporter in the resistant parasites showing that the Berenil K_i increased greatly ($>10\,\mu$M, Table 2), and that there was a major modification of the binding properties of the P2 transporter in the drug-resistant parasites. The changes in the K_i values of adenosine and Berenil highlight the significant changes in the binding affinities of P2 associated with the development of resistance.

DISCUSSION

Transport of $5\,\mu$M adenosine was followed for 120 sec in a drug-sensitive *T. evansi* clone and in its Mel CY-resistant clone. The striking changes we observed were in the P2 transporter where there was a decreased maximum uptake capacity (A_{max}) from $112{\cdot}5 \pm 17{\cdot}8$ pmol/10^8 cells to $33{\cdot}6 \pm 8{\cdot}5$ pmol/10^8 cells, a decrease in maximum uptake rate (V_{max}) from $28{\cdot}9 \pm 5{\cdot}1$ pmol/10^8 cells/sec to $3{\cdot}3 \pm 1{\cdot}1$ pmol/10^8 cells/sec, an increase in the affinity of the P2

https://doi.org/10.1017/S003118200300413X Published online by Cambridge University Press

transporter for adenosine, but a decrease in the affinity for adenine and Berenil.

Changes in A_{max} *associated with resistance*

The A_{max} of a transporter defines its maximum uptake capacity of adenosine and is a function of (i) the number (concentration) of the transporter molecules available; (ii) the concentration of the substrate and affinity of the substrate for the transporter; and (iii) the rate of removal and metabolism of adenosine. In our experiments on the P2 transporter, 5 μM adenosine was found to provide saturable conditions for maximum uptake by these trypanosomes; thus A_{max} was not limited by substrate concentration. We measured the time constant (T: see Materials and Methods section) that estimates the reciprocal of the combined rate of metabolism and elimination and found that in the drug-sensitive trypanosomes (WT) that the time-constant was $57{\cdot}7 \pm 10{\cdot}3$ sec and was not significantly changed in the drug-resistant clone, CR3.1 ($72{\cdot}8 \pm 9{\cdot}2$ sec). We found no evidence of a change in the combined estimate of the rate of removal and metabolism. The change in A_{max} we observed can be explained by a decrease in the numbers of individual transporter molecules. We show in the next paragraph that the maximum rate of adenosine uptake (V_{max}), a more direct measure of the number of transporter molecules, is reduced for the P2 transporter. Since Mel CY is taken up on the P2, a reduced number of P2 transporter molecules, reduces substrate accumulation via the P2 transporter and provides a mechanism for resistance to Mel CY.

V_{max}, *P1 and P2*

The V_{max} that we obtained for the P1 transporter in drug-sensitive *T. evansi* (12·4 pmol/10^8 cells/sec) is comparable to the V_{max} reported for *T. brucei* (10·6 pmol/10^8 cells/sec; Carter & Fairlamb, 1993) and *T. equiperdum* (8·4 pmol/10^8 cells/sec; Barrett *et al*. 1995). Similarly, the K_m for P1 transport in *T. evansi* (0·07 μM) was similar to that reported for the P1 transporter in *T. brucei* (0·15 μM; Carter & Fairlamb, 1993). A higher K_m (0·60 μM) was reported for *T. equiperdum* (Barrett *et al*. 1995).

Our V_{max} for the P2 transporter (28·9 pmol/10^8 cells/sec) in the drug-sensitive *T. evansi* was higher than reported values for either *T. brucei* (9·5 pmol/10^8 cells/sec) or *T. equiperdum* (6·9 pmol/10^8 cells/sec). The higher values of V_{max} and A_{max} at the P2 are consistent with our earlier report (Suswam *et al*. 2001) that the P2 of *T. evansi* is the larger of the two transporter systems. Hence the relative P2 : P1 capacity of *T. evansi* is different from that of *T. brucei* and *T. equiperdum*. This greater significance of the P2 of *T. evansi* provides an advantage for effective exploitation as a chemotherapeutic target.

In contrast, the value for V_{max} at the P2 in the drug-resistant trypanosomes was decreased 10-fold. In related studies on purine transport in other trypanosomatids (de Koning *et al*. 2000), observed increased V_{max} of adenosine in *Crithidia luciliae*, which was abolished by treatment with cycloheximidine (inhibits protein synthesis). These authors suggested that increased V_{max} was due to increased numbers of transporters. In our study the decreased V_{max} coupled with the marked decrease in A_{max} and K_i seen in the drug-resistant parasites is confirmation that the reduced uptake at the P2 is a result of reduced numbers of P2 transporter molecules. The K_m for adenosine at the P2 transporter in drug sensitive *T. evansi* (0·74 μM) determined in this study was also very similar to those reported for *T. brucei* (0·59 μM) and *T. equiperdum* (0·7 μM).

Change in affinities of the P2 transporter and reduction of adenosine uptake in drug-sensitive T. evansi

An earlier report (Barrett *et al*. 1995) suggested that reduced adenosine uptake at the P2 transporter may be a result of decreased affinity for substrate. Unfortunately, in that report the K_m of adenosine on the P2 transporter of the drug-resistant *T. equiperdum* could not be determined. The data presented here show that the K_m for adenosine on the P2 was decreased in the resistant clone showing, interestingly, that there was an increase in affinity for adenosine on the P2 transporter. The decrease in the maximum rate of uptake (V_{max}) of adenosine uptake that we observe is therefore explained by a down-regulation of the P2 transporter molecules, not a decrease in affinity. The inhibition profiles for adenine and Berenil showed that the P2 transporter did change its binding characteristics: a significant increase in inhibition constant (K_i) for adenine was found on this transporter; and Berenil failed to interact with the P2 transporter. These observations suggest conformational changes at the P2 binding site. The apparent changes in the residual P2 transporter activity may indicate a selection of a new transporter isotype of the P2 system that has increased affinity for adenosine but has low affinity for adenine and does not recognize Berenil as substrate. The presence of purine transporter isotypes with similar but distinct activities has been documented in *T. brucei* (Sanchez *et al*. 2002).

In conclusion, our study confirms the existence of 2 distinct adenosine transport systems in *T. evansi*, consistent with our earlier report (Suswam *et al*. 2001), and with the general trend in other trypanosomatids (Carter & Fairlamb, 1993; Barrett *et al*. 1995; Scott *et al*. 1997). We also observed evidence that there is a change in binding of the P2 transporter associated with the development of resistance

https://doi.org/10.1017/S003118200300413X Published online by Cambridge University Press

to Mel CY and this change is seen in addition to the down-regulation of the P2 transporter. Our observations also establish the basis for resistance to Berenil, and lend weight to earlier observations of Barrett *et al*. (1995) on Berenil-resistant *T. equiperdum*.

We are pleased to acknowledge the support of the NIH to RJM: RO1 A147194-02.

REFERENCES

BARRETT, M. P., ZHANG, Z. Q., DENISE, H., GIROUD, C. & BALTZ, T. (1995). A diamidine-resistant *Trypanosoma equiperdum* clone contains a P2 purine transporter with reduced substrate affinity. *Molecular and Biochemical Parasitology* **73**, 223–229.

CARTER, N. S., BERGER, B. J. & FAIRLAMB, A. H. (1995). Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and resistant *Trypanososma brucei brucei*. *The Journal of Biological Chemistry* **270**, 28153–28157.

CARTER, N. S. & FAIRLAMB, A. (1993). Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature, London* **361**, 173–175.

GIBALDI, M. (1991). *Biopharmaceutics and Clinical Pharaceutics*, 4th Edn. Lea and Febiger. London.

DE KONING, H. P. & JARVIS, S. M. (1997). Purine nucleobase transport in bloodstream forms of *Trypanosoma brucei* is mediated by two novel transporters. *Molecular and Biochemical Parasitology* **89**, 245–258.

DE KONING, H. P. & JARVIS, S. M. (1998). A highly selective, high-affinity transporter for uracil for *Trypanosoma brucei brucei*: evidence for proton-dependent transport. *Biochemical and Cell Biology* **76**, 853–858.

DE KONING, H. P. & JARVIS, S. M. (1999). Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: Substrate recognition motifs and affinity for trypanocidal drugs. *Molecular Pharmacology* **56**, 1162–1170.

DE KONING, H. P., WATSON, C. J. & JARVIS, S. M. (1998). Characterization of a nucleoside/proton symporter in procyclic *Trypanosoma brucei brucei*. *The Journal of Biological Chemistry* **273**, 9486–9494.

DE KONING, H. P., WATSON, C. J., SUTCLIFF, L. & JARVIS, S. M. (2000). Differential regulation of nucleoside and nucleobase transporters in *Crithidia fasciculata* and *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **106**, 93–107.

FAIRLAMB, A. H., CARTER, N. S., CUNNINGHAM, M. & SMITH, K. (1992). Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology* **53**, 213–222.

MASER, P., SUTERLING, C., KRALLI, A. & KAMINSKY, R. (1999). A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **285**, 242–244.

ROSS, C. A. & BARNS, A. M. (1996). Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Tryanosoma evansi*. *Parasitology Research* **82**, 183–188.

SANCHEZ, M. A., TRYON, R., GREEN, J., BOOR, I. & LANDFEARS, S. M. (2002). Six related nucleoside/nucleobase transporters from *Trypanosoma brucei* exhibit distinct biochemical functions. *The Journal of Biological Chemistry* **277**, 21499–21504.

SCOTT, A. G., TAIT, A. & TURNER, C. M. R. (1997). *Trypanosoma brucei*: lack of cross-resistance to melarsoprol *in vitro* by Cymelarsan-resistant parasites. *Experimental Parasitology* **86**, 181–190.

SUSWAM, E. A., TAYLOR, D. W., ROSS, C. A. & MARTIN, R. J. (2001). Changes in properties of adenosine transporters in *Trypanosoma evansi* and modes of selection of resistance to the melaminophenyl arsenical drug, Mel Cy. *Veterinary Parasitology* **102**, 193–208.