Uptake of the antitrypanosomal drug 5'-[(Z)-4-amino-2-butenyl]methylamino-5'-deoxyadenosine (MDL 73811) by the purine transport system of *Trypanosoma brucei brucei*

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An irreversible inhibitor of *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), 5'-[(Z)-4-amino-2-butenyl]methylamino-5'-deoxyadenosine (MDL 73811), was found to cure *Trypanosoma brucei brucei* and multidrug-resistant *T. b. rhodesiense* infections in mice [Bitonti, Byers, Bush, Casara, Bacchi, Clarkson, McCann & Sjoerdsma (1990) Antimicrob. Agents Chemother. 34, 1485–1490]. Doses of this drug which resulted in a rapid clearance of parasites from *T. b. brucei*-infected rats resulted in plasma levels of 50–60 μM -MDL 73811 and an intratrypanosomal MDL 73811 concentration of 1.9 mM within 10 min of administration [Byers, Bush, McCann & Bitonti (1991) Biochem. J. 274, 527–533]. Based on this finding we speculated that MDL 73811, which is an adenosine analogue, is a substrate for the trypanosome active purine transport system. We now report evidence that supports this hypothesis. MDL 73811 uptake by *T. b. brucei* *in vitro* was time- and temperature-dependent and was saturable over a time course in which MDL 73811 metabolism was undetectable, suggesting that MDL 73811 uptake is a transport-mediated phenomenon. Inhibition of MDL 73811 uptake by purine nucleosides is consistent with the drug being a substrate for the trypanosome purine transport system. The accumulation of MDL 73811 by cultured L1210 mouse leukaemia cells was significantly less than by trypanosomes exposed to the same pharmacologically relevant concentrations of MDL 73811. Given that the half-life of MDL 73811 in the plasma of rats and mice is approx. 10 min, it seems likely that the existence of a highly active parasite transport system for MDL 73811 is crucial for the sensitivity of trypanosomes towards MDL 73811 *in vivo*, and that the absence of active transport of MDL 73811 by the host's cells may play a role in the selectivity of this drug.

INTRODUCTION

Administration of 5'-[(Z)-4-amino-2-butenyl]methylamino-5'-deoxyadenosine (MDL 73811), an enzyme-activated irreversible inhibitor of *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), a key enzyme in the synthesis of spermidine, cures mice infected with the African trypanosome *Trypanosoma brucei brucei* (Bitonti *et al.*, 1990). We reported recently that administration of MDL 73811 to *T. b. brucei*-infected rats results in a 70% decrease in parasitaemia within 1 h, and a complete disappearance of parasites by 5 h (Byers *et al.*, 1991). The decrease in parasitaemia was preceded by complete inhibition of AdoMetDC activity by 10 min after injection of MDL 73811. The half-life of the drug and the peak plasma concentration after a single intraperitoneal injection of 50 mg of MDL 73811/kg, a dose which results in a rapid decrease in parasitaemia, were found to be approx. 10 min and 50 μM respectively. The intracellular concentration of MDL 73811 in trypanosomes 10 min after injection with 25 mg of the drug/kg was found to be 1.9 mM. Given the low plasma concentration of this drug, the high intracellular concentration of MDL 73811 suggested that trypanosomes have an active transport system for which MDL 73811 is a substrate. Because the half-life of MDL 73811 is approx. 10 min, the existence of a highly active transport system is likely to be crucial for the sensitivity of the trypanosomes towards MDL 73811 *in vivo*.

Many protozoan parasites, including African trypanosomes, cannot synthesize purines (reviewed in Hammond & Gutteridge,

1984) and, therefore, they are dependent on exogenous purines and have a specific, active transport system for purine nucleosides (Hammond & Gutteridge, 1984; Gottlieb & Dwyer, 1988). Because MDL 73811 is an adenosine analogue, we hypothesized previously that the purine nucleoside transport system may be involved in the uptake of MDL 73811 by trypanosomes (Byers *et al.*, 1991). Examination of the uptake of [^3H]MDL 73811 by trypanosomes has now provided direct evidence that MDL 73811 is taken up by an active nucleoside transport system.

MATERIAL AND METHODS

Chemicals

MDL 73811, MDL 74281 (5-methylamino-5'-deoxyadenosine; Casara *et al.*, 1989) and MDL 73581 (5'-[(Z)-4-amino-2-butenyl]ethylamino-5'-deoxyadenosine) were synthesized at the Marion Merrell Dow Research Institute. MDL 74344 {5'-[(4-amino-2-butenyl)methylamino]-5'-deoxyadenosine}, the ethynyl analogue of MDL 73811, was synthesized at the Marion Merrell Dow Research Institute and was tritiated by New England Nuclear to produce [^3H]MDL 73811.

[^3H]MDL 73811 was purified by passing the tritiated material from New England Nuclear on to a pre-column packed with Partisil 10-SCX (4.6 mm $\times$ 100 mm) and then on to a Whatman Partisil 10-SCX (4.6 mm $\times$ 250 mm) column using a 0.25 M-ammonium formate (pH 4) buffer containing 10% acetonitrile delivered at 2 ml/min at 35 $^{\circ}\text{C}$. Fractions (1 ml) were collected and the elution of MDL 73811 was monitored at 254 nm using

Abbreviations used: AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; MDL 73811, 5'-[(Z)-4-amino-2-butenyl]methylamino-5'-deoxyadenosine; MDL 74281, 5-methylamino-5'-deoxyadenosine; MDL 73581, 5'-[(Z)-4-amino-2-butenyl]ethylamino-5'-deoxyadenosine; MDL 74344, 5'-[(4-amino-2-butenyl)methylamino]-5'-deoxyadenosine.

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a u.v. detector. Fractions corresponding to the authentic MDL 73811 peak were evaporated to dryness and redissolved in water. Aliquots of these fractions were then assayed for purity as described below. Fractions which when analysed resulted in single u.v. and radioactive peaks corresponding to the MDL 73811 standard were pooled and again assayed to determine the concentration (20.55 μM) and specific radioactivity of the [^3H]MDL 73811 (15000 $\mu\text{Ci}/\mu\text{mol}$).

All other chemicals were from Sigma and were of the highest purity available.

Trypanosomes

T. b. brucei (strain Lab 110/EATRO) was maintained by syringe passage in male Sprague-Dawley rats. Trypanosomes were collected in blood, via cardiac puncture using EDTA as an anticoagulant, from infected male Sprague-Dawley rats which were anaesthetized with CO_2 . Trypanosomes were separated from blood cells by passage over a DEAE-cellulose column (Lanham & Godfrey, 1970) equilibrated with 90 mM-Tris/HCl, pH 7.8, containing 50 mM-NaCl and 2% glucose (TSG), and then washed several times in TSG before use in uptake studies.

Separation and quantification of MDL 73811

MDL 73811 was separated and quantified as described previously (Byers *et al.*, 1991). A reverse-phase chromatography system using an h.p.l.c. apparatus from Beckman Instruments was used to separate MDL 73811. The sample was passed first over a Waters Bondpak C_{18} pre-column and then on to a Beckman Ultrasphere Ion Pairing 5 μm C_{18} (250 mm $\times$ 4.6 mm) column (Byers *et al.*, 1991) using a sodium phosphate buffer system described by Wagner *et al.* (1982). U.v. absorption at 254 nm was used to detect MDL 73811 and an on-line radioactivity detector from Radiomatic was used to detect and quantify ^3H in the sample.

Uptake of MDL 73811 by trypanosomes and L1210 cells

Purified trypanosomes were washed twice in Dulbecco's modified Eagle's medium (pH 7.5; Gibco) supplemented with 2 mM-glutamine, 0.2% glucose, 12 μg of penicillin/ml, 12 μg of streptomycin/ml and 30 mM-Hepes, and then suspended in this medium supplemented with 1% BSA (Fraction V; CalBiochem) at 2×10^9 trypanosomes/ml in Microfuge tubes. This cell suspension was incubated in a shaking water bath at 37 °C for 1–3 min prior to dilution in the same medium containing a $2 \times$ final concentration of [^3H]MDL 73811 and, where indicated, a $2 \times$ final concentration of a potential inhibitor of MDL 73811 uptake. After further incubation in a shaking water bath at 37 °C, unlabelled MDL 73811 was added to give a final concentration of 1 mM (except where the MDL 73811 content was to be quantified by u.v. absorption after separation by h.p.l.c.), and the trypanosomes were quickly sedimented in a Microfuge at 4 °C and then washed twice with ice-cold TSG. The bottom of the Microfuge tube, which contained the washed cell pellet, was cut off and placed in a scintillation vial containing 10 ml of scintillant (Ready Protein; Beckman), and the [^3H]MDL 73811 content of the pellets was determined in a scintillation counter.

The method used to determine MDL 73811 uptake by L1210 mouse leukaemia cells was identical to that described above. L1210 cells were grown as suspension cultures in RPMI 1640 medium (Gibco) supplemented with 10% Nu-Serum (Collaborative Research Inc.), 16 mM-Hepes, 7.8 mM-Mops, 12 μg of penicillin/ml and 12 μg of streptomycin/ml at 37 °C in air/ CO_2 (19:1) and 90% humidity. Cultured L1210 cells were washed twice in phosphate-buffered saline [137 mM-NaCl, 3 mM-KCl, 16 mM- Na_2HPO_4 , 0.2 mM- KH_2PO_4 , CaCl_2 (0.2 mg/l),

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 mg/l)] as described for the trypanosomes. L1210 cells were suspended at 8×10^6 cells/ml prior to dilution with medium containing [^3H]MDL 73811. A volume of 0.675 pl/cell was used to calculate the intracellular concentration of MDL 73811 in L1210 cells (R. Poulin and A. E. Pegg, personal communication).

RESULTS

Uptake of MDL 73811 into trypanosomes was both time- and temperature-dependent (Fig. 1). Uptake at 37 °C was approximately linear (correlation coefficient = 0.988) for up to 7 min. Uptake of the drug at 4 °C proceeded at a much lower rate than at 37 °C, and the uptake at the lower temperature appeared to be linear for at least 5 min. Trypanosomes exposed to 40 μM -MDL 73811 for 5 min at 37 °C were > 95% viable, as determined by microscopic observation. The remainder of the uptake experiments were carried out at 37 °C over a time period of 5 min or less.

Trypanosome uptake of MDL 73811 was saturable (Fig. 2), which is consistent with the uptake being a transport phenomenon. The apparent K_m for MDL 73811 uptake was calculated to be approx. 5 μM by a Lineweaver-Burk analysis. In one experiment the intracellular content of MDL 73811 in trypanosomes was determined by scintillation counting and by u.v. absorption after separation by reverse-phase h.p.l.c. The intracellular concentration of MDL 73811 calculated by both methods was virtually identical (Table 1), indicating that trypanosomes do not metabolize the drug over the course of an uptake experiment. Uptake of MDL 73811 by L1210 cells was linear over a concentration range of 0.01 to 100 μM (correlation coefficient of 1.0 for uptake during a 5 min exposure to 0.01, 0.1, 1.0, 10 or 100 μM -MDL 73811). The intracellular concentration of MDL 73811 of these cells was markedly less than that in trypanosomes exposed to the same pharmacologically relevant MDL 73811 concentrations.

Fig. 1. Time- and temperature-dependent uptake of [^3H]MDL 73811 by *T. b. brucei*

Trypanosomes were harvested, purified, washed, counted on a haemocytometer and then placed in Microfuge tubes in the presence of 2 μM -[^3H]MDL 73811 at either 37 °C (●) or 4 °C (○). After the indicated period of time, unlabelled MDL 73811 was added to give a final concentration of 1 mM-MDL 73811 and the trypanosomes were quickly sedimented and washed twice with TSG at 4 °C. The amount of MDL 73811 taken up was calculated based on the radioactive content of the cell pellet and either the number of trypanosomes in the assay or a trypanosome volume of 58 $\mu\text{l}/10^9$ trypanosomes. The values given are the means $\pm$ s.d. of three assays.

Fig. 2. Saturable uptake of [^3H]MDL 73811 by *T. b. brucei*

(a) Trypanosomes were harvested, purified, washed, counted on a haemocytometer and then placed in Microfuge tubes in the presence of the indicated concentration of [^3H]MDL 73811 (0.1, 0.3, 1.0, 3.0, 10 or 30 μM) and incubated at 37 $^{\circ}\text{C}$ for 3 min. After this incubation unlabelled MDL 73811 was added to give a final concentration of 1 mM-MDL 73811, and the trypanosomes were quickly sedimented and washed twice with TSG at 4 $^{\circ}\text{C}$. The amount of MDL 73811 taken up was calculated based on the radioactive content of the cell pellet and the number of trypanosomes in the assay. The values given are the means $\pm$ S.D. of three assays. (b) Lineweaver-Burk plot of the uptake data shown in (a).

MDL 74281 is a metabolic product produced by the catalytic action of AdoMetDC on MDL 73811 during inhibition of that enzyme (Casara *et al.*, 1989), and MDL 73581, which is an analogue of MDL 73811, is an inhibitor of AdoMetDC (Fig. 3). MDL 73811 uptake was inhibited by MDL 74281 and MDL 73581 (Fig. 4a). MDL 73811 uptake was also inhibited by the purine nucleosides adenosine and guanosine, but not by the pyrimidine nucleoside cytosine (Fig. 4a). Adenine and guanine were also inhibitors of this uptake (Fig. 4b), although these purine bases did not inhibit as well as their respective nucleosides. The nucleoside analogue nitrobenzylthioinosine, an inhibitor of nucleoside transport in mammalian cells, was a very poor inhibitor of MDL 73811 uptake relative to the natural nucleosides (Fig. 4b). The inhibition of MDL 73811 uptake by the structural analogues increased the K_m of uptake without altering the V_{max} , and therefore is consistent with competitive inhibition.

DISCUSSION

It appears clear that MDL 73811 enters *T. b. brucei* via a transport-mediated mechanism. The uptake of this drug by the trypanosomes was saturable, temperature-sensitive and inhibited by synthetic and natural structural analogues, all of which are

Table 1. Accumulation of [^3H]MDL 73811 by *T. b. brucei* and L1210 cells

The Table shows a comparison of intratrypanosomal MDL 73811 quantification by radioactive and non-radioactive methods and a comparison of accumulation in *T. b. brucei* and L1210 cells *in vitro*. Trypanosomes were harvested, purified, washed, counted in a haemocytometer and then placed in Microfuge tubes in the presence of the indicated concentration of [^3H]MDL 73811 and incubated at 37 $^{\circ}\text{C}$ for 5 min. After incubation the trypanosomes were quickly sedimented and washed twice with TSG at 4 $^{\circ}\text{C}$. The cell pellet was suspended in 10% trichloroacetic acid and the protein was precipitated and removed by centrifugation. Aliquots of the supernatant were added to scintillation cocktail and counted for radioactivity using a scintillation counter or loaded on to an h.p.l.c. column for separation of MDL 73811, which was quantified using a u.v. detector. The minimum level of detection by h.p.l.c./u.v. was 4 μM . L1210 cells were washed, counted in a haemocytometer and then placed in Microfuge tubes in the presence of 0.01, 0.1, 1, 10 or 100 μM -[^3H]MDL 73811 and incubated at 37 $^{\circ}\text{C}$ for 5 min. After incubation the L1210 cells were prepared for scintillation analysis using the protocol for the trypanosomes given above. Because the accumulation in L1210 cells was linear (correlation coefficient = 1.000) with respect to extracellular MDL 73811 concentration, we extrapolated the accumulation of the drug by L1210 cells exposed to 0.3, 3, 20, 30 and 40 μM -MDL 73811 for comparison with accumulation by the trypanosomes. The values listed are the means $\pm$ S.D. for three assays. n.d., not determined.

[^3H]MDL 73811 concn. (μM)	Intracellular MDL 73811 (μM)		
	<i>T. b. brucei</i>		
	Scintillation counting	H.p.l.c./u.v.	L1210 cells
0.01	n.d.	n.d.	0.02 $\pm$ 0.001
0.1	0.74 $\pm$ 0.03	< 4.0	0.19 $\pm$ 0.02
0.3	2.1 $\pm$ 0.3	4.5 $\pm$ 0.5	0.39*
1	7.0 $\pm$ 0.2	5.9 $\pm$ 0.9	1.5 $\pm$ 0.1
3	19 $\pm$ 1	19 $\pm$ 3	4.5*
10	63 $\pm$ 4	53 $\pm$ 7	15 $\pm$ 1.5
20	98 $\pm$ 4	96 $\pm$ 13	28*
30	112 $\pm$ 4	115 $\pm$ 2	42*
40	139 $\pm$ 11	143 $\pm$ 13	56*
100	n.d.	n.d.	140 $\pm$ 15

* Extrapolated value.

Fig. 3. Chemical structures of MDL 73811, MDL 73581 and MDL 74281

Fig. 4. Inhibition of [^3H]MDL 73811 uptake by synthetic structural analogues and by purine nucleosides and bases

Trypanosomes were harvested, purified, washed, counted on a haemocytometer and then placed in Microfuge tubes in the presence of (a) [^3H]MDL 73811 (0.1, 0.3, 1.0 and 3.0 μM) and 100 μM -MDL 74281 (●), -MDL 73581 (▽), -adenosine (■), -guanosine (□), or -cytosine (○) (▼, control), or (b) [^3H]MDL 73811 (0.1, 0.3, 1.0, 3.0 μM) and 200 μM -adenine (●), -guanine (▽) or -nitrobenzylthioinosine (○) (○, control), and incubated at 37 °C for 5 min. After this incubation, unlabelled MDL 73811 was added to give a final concentration of 1 mM-MDL 73811 and the trypanosomes were quickly sedimented and washed twice with TSG at 4 °C. The amount of MDL 73811 taken up was calculated based on the radioactive content of the cell pellet and the number of trypanosomes in the assay. The inverse of the mean for three assays is presented in this Figure.

characteristics of uptake facilitated by transport. The finding that intracellular MDL 73811 was not metabolized over the 5 min time course of the uptake experiment excludes the possibility that the saturable uptake observed was due to diffusion limited by a metabolism-generated gradient. The observation that MDL 73811 was concentrated greatly in trypanosomes (Byers *et al.*, 1991) implies that this transport utilizes metabolic energy. Our hypothesis that MDL 73811 is a substrate for the purine transport system of trypanosomes was supported by the finding that purine bases and nucleosides competitively inhibited uptake of this drug.

Aronow *et al.* (1987) reported the existence of two independent transport systems in *Leishmania donovani* promastigotes (an organism related closely to *T. b. brucei*), one which facilitates the uptake of inosine, guanosine and their analogues, and another which transports adenosine and its analogues as well as pyrimidine nucleosides. Our data do not support the existence of similar transport systems in trypanosomes. While the purine nucleosides adenosine and guanosine both inhibited MDL 73811

uptake by *T. b. brucei*, this uptake was not inhibited by the pyrimidine nucleoside cytidine, which is reported to be a substrate for the guanosine transporter in *L. donovani*. The observation that the purine bases were relatively poor competitive inhibitors for MDL 73811 uptake suggests that the system which transports MDL 73811 has a higher affinity for nucleosides than for the bases themselves. The finding that MDL 73811 uptake by *T. b. brucei* was very poorly inhibited by nitrobenzylthioinosine, an inhibitor of mammalian purine nucleoside transport (Lauzon & Paterson, 1977), and our observation that MDL 73811 appears to enter mammalian cells via diffusion, highlight differences between the purine transport systems of African trypanosomes and their mammalian hosts. The relatively poor accumulation of MDL 73811 in mammalian cells may explain, in part, the selectivity of MDL 73811 against trypanosomes. Parasites in *T. b. brucei*-infected rats are rapidly cleared from the blood of rats treated with as little as 25 mg of MDL 73811/kg, while these animals show no obvious adverse reactions to this treatment (Byers *et al.*, 1991) and the LD₅₀ for MDL 73811 in mice is over 300 mg/kg (T. L. Byers, P. Casara & A. J. Bitonti, unpublished work).

The inability of many parasitic protozoa to synthesize purines *de novo* has long been appreciated, and has resulted in the targeting of this deficiency in the design of chemotherapeutic agents for specific parasitic diseases (Marr & Berens, 1983). Although the uptake of purines and their bases by parasitic protozoa has not been extensively studied, it is generally accepted that this uptake is due to an active transport system developed by these organisms to exploit the host. Our data indicate that we have successfully exploited the dependence of African trypanosomes on exogenous purine sources to deliver the antitrypanosomal agent MDL 73811 to the target organism. MDL 73811 concentrations in trypanosomes were found to be over 30-fold higher than those in host plasma within 10 min after intraperitoneal injection of infected rats with a minimal effective antitrypanosomal dose. Since the half-life of MDL 73811 in these animals was less than 10 min, it is likely that the uptake of the drug by an active mechanism by the trypanosome purine transport system may be crucial for the sensitivity of the trypanosomes towards MDL 73811. In addition, the active transport of MDL 73811 by trypanosomes, the short plasma half-life of this drug and the relatively low accumulation of MDL 73811 by mammalian cells probably play a role in the selective antitrypanosomal effects of MDL 73811.

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