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Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in *Trypanosoma brucei brucei* *S*-adenosyl-L-methionine

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We reported recently that 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; EC 4.1.1.50), a key enzyme in the synthesis of spermidine, cures African trypanosome infections in mice. The precise mechanism of action of MDL 73811 was not clear because a rapid disappearance of trypanosomes from the bloodstream of treated rats occurred before significant depletion of spermidine. Administration of MDL 73811 to *Trypanosoma brucei brucei*-infected rats resulted in a 70% decrease in parasitaemia within 1 h and a complete disappearance of parasites by 5 h. The reduction in parasitaemia was accompanied by complete inhibition of AdoMetDC activity by 10 min after injection of MDL 73811; inhibition was sustained for at least 4 h. Polyamine levels in trypanosomes were unaffected during the first 1 h in which the marked decrease in parasitaemia was observed, but parasite AdoMet levels increased 20-fold within this time. In contrast, exposure of cultured mammalian cells to MDL 73811 resulted in only a 1.5–2-fold increase in AdoMet levels over a 6 h time course. Experiments with inhibitors of ornithine decarboxylase (ODC) also suggested that the increased AdoMet levels might be an important factor for antitrypanosomal efficacy. Trypanosomes taken from rats treated for 36 h with eflornithine, an inhibitor of ODC, were depleted of putrescine and had markedly decreased spermidine levels. These organisms also had less than 10% of control AdoMetDC activity, and had elevated decarboxy AdoMet (> 4000-fold) and AdoMet (up to 50-fold) levels. The methyl ester of α-monofluromethyl-3,4-dehydro-ornithine (Δ-MFMO-CH_3), which cures murine *T. b. brucei* infections, and the ethyl ester analogue of this compound (Δ-MFMO-C_2H_5), which does not cure this infection, become ODC inhibitors upon hydrolysis and thus were tested for their effects on trypanosomal polyamines, AdoMet and decarboxy AdoMet levels. Although both esters of Δ-MFMO depleted trypanosomal polyamines, AdoMet and decarboxy AdoMet levels were elevated in *T. b. brucei* from infected mice treated with Δ-MFMO-CH_3 but not in parasites from mice treated with the Δ-MFMO-C_2H_5. These data suggest that inhibition of AdoMetDC, either directly with MDL 73811 or indirectly with inhibitors of ODC, apparently leads to a trypanosome-specific elevation of AdoMet. It is possible that major changes in AdoMet, rather than changes in polyamines, may be responsible for the antitrypanosomal effects of these drugs.

INTRODUCTION

Inhibition of putrescine biosynthesis by eflornithine (α-difluromethylornithine), a specific enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), inhibits replication of a number of parasitic protozoa (Bacchi & McCann, 1987) and is effective for the treatment of human West African trypanosomiasis (Sjoerdsma & Schechter, 1984; Van Nieuwenhove *et al.*, 1985; Schechter *et al.* 1987). Trypanosome *S*-adenosyl-L-methionine decarboxylase (AdoMetDC; EC 4.1.1.50), a key enzyme in the synthesis of spermidine from putrescine and *S*-adenosyl-L-methionine (AdoMet) (Fig. 1), is inhibited by the antitrypanosomal agents methylgloxal bis(guanylhydrazone) (MGBG), Berenil (diminazene aceturate) and pentamidine *in vitro*, lending further support to the hypothesis that interference with polyamine metabolism is a target for designing other antitrypanosomal agents (Bitonti *et al.*, 1986). As a result, a recently synthesized, potent, enzyme-activated, irreversible, inhibitor of AdoMetDC, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (MDL 73811), was tested and was found to cure *Trypanosoma brucei brucei* and multidrug-resistant *T. b.*

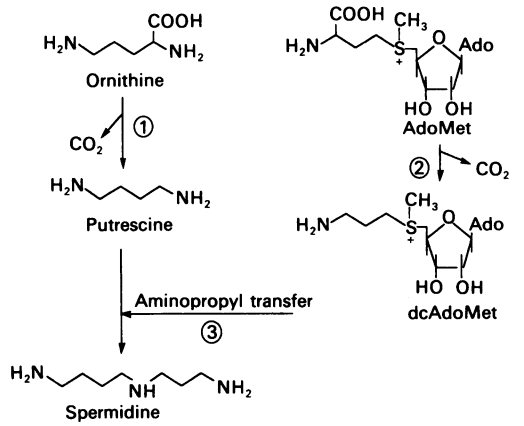

Fig. 1. Polyamine biosynthetic pathway in African trypanosomes

The following enzymes are involved: 1, ODC; 2, AdoMetDC; 3, spermidine synthase. Note that mammalian cells synthesize spermine from spermidine via spermine synthetase, an aminopropyltransferase which specifically transfers the aminopropyl group of dcAdoMet to spermidine.

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; dcAdoMet, decarboxylated *S*-adenosyl-L-methionine; MDL 73811, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine; MDL 74281, 5′-methylamino-5′-deoxyadenosine; Δ-MFMO-CH_3, α-monofluromethyl-3,4-dehydro-ornithine methyl ester; Δ-MFMO-C_2H_5, α-monofluromethyl-3,4-dehydro-ornithine ethyl ester; MGBG, methylglyoxal bis(guanylhydrazone); MTA, methylthioadenosine; ODC, ornithine decarboxylase; SAH, *S*-adenosyl-L-homocysteine.

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rhodesiense infections in mice, and was at least 100-fold more potent than eflornithine against these organisms (Bitonti *et al.*, 1990). However, the changes in putrescine and spermidine observed in trypanosomes treated with MDL 73811 were not of the magnitude that would be expected to be responsible for the disappearance of parasites from the blood of infected rats, which occurred within 4 h of treatment with MDL 73811. Thus another mechanism of action other than polyamine depletion was sought.

Several mechanisms have been suggested previously to explain the marked and selective antitrypanosomal effects of eflornithine compared with the effects of the drug in mammalian cells, including slow turnover of trypanosome ODC relative to mammalian ODC (Phillips *et al.*, 1987), depletion of the trypanosomatid spermidine-containing enzyme cofactor trypanothione (Fairlamb & Cerami, 1985; Fairlamb *et al.*, 1987) and a greater dependence on spermidine for trypanosome division because of the high doubling rate (< 6 h) of the parasite. However, none of these possible mechanisms would seem to satisfactorily explain the rapid trypanocidal action of MDL 73811. Two additional biochemical consequences of the inhibition of ODC have been reported which appear to be unique to trypanosomes. After treatment with eflornithine, AdoMetDC activity was significantly inhibited (Bacchi *et al.*, 1983), and AdoMet levels were elevated approx. 50-fold (Yarlett & Bacchi, 1988). The changes in AdoMetDC and AdoMet are undoubtedly amongst the most remarkable biochemical changes in trypanosomes treated with eflornithine.

We now show that direct inhibition of AdoMetDC with MDL 73811 in African trypanosomes results in elevated levels of AdoMet. Furthermore, we suggest that this rapid and massive increase in AdoMet may be specific to the parasite, and propose that this change, rather than polyamine depletion, may be either directly or indirectly responsible for the antitrypanosomal action of the drug. We also propose that the specific antitrypanosomal action of ODC inhibitors may also be dependent on indirect elevation of AdoMet via depletion of putrescine.

MATERIALS AND METHODS

Chemicals

MDL 73811, MDL 74281, α-monofluromethyl-3,4-dehydro-ornithine methyl ester (Δ-MFMO-CH_3), α-monofluromethyl-3,4-dehydro-ornithine ethyl ester (Δ-MFMO-C_2H_5) and eflornithine were synthesized at the Merrell Dow Research Institute (Metcalf *et al.*, 1978; Mamont *et al.*, 1986; Casara *et al.*, 1989). *S*-Adenosyl-[*carboxy*-^{14}C]-L-methionine (56.9 mCi/mmol) was obtained from New England Nuclear. AdoMet was from Boehringer Mannheim. All other chemicals were from Sigma and were of the highest purity available.

Trypanosomes

T. b. brucei (strain Lab 110/EATRO) were maintained by syringe passage into male Sprague–Dawley rats. Parasitaemia was monitored by collecting 3–5 μl of blood from small tail cuts, diluting sequentially with 0.83% NH_4Cl and with a solution of 90 mM-Tris/HCl, pH 7.8, containing 50 mM-NaCl and 2% glucose (TSG buffer), and then counting the trypanosomes in a haemocytometer. Where parasitaemia in individual animals was monitored over time, the initial tail cut was made at the tip of the tail and sequential cuts were made linearly towards the base of the tail. Trypanosomes for biochemical studies were collected in blood, via cardiac puncture using EDTA as an anticoagulant, from infected male Sprague–Dawley rats or male CD-1 mice which were anaesthetized with CO_2. Trypanosomes were separated from blood cells by passage over a DEAE-cellulose column equilibrated with TSG (Lanham & Godfrey, 1970) and then washed several times in TSG before preparation for biochemical analysis.

Trypanosome extracts and AdoMetDC analysis

Purified trypanosomes were suspended in a solution of 50 mM-sodium phosphate (pH 7.4) containing 2 mM-dithiothreitol, 3 mM-putrescine and 0.1 mM-EDTA and disrupted on ice with 3 × 15 s periods of continuous sonication (setting 3, Branson Sonifier) interrupted by 30 s intervals without sonication. Disrupted trypanosomes were centrifuged for 30 min at 10000 *g* in a refrigerated (4 °C) centrifuge and the supernatant fraction, containing 7–10 mg of protein/ml, was removed and stored frozen at −70 °C for up to 1 month without loss of activity.

The assay for AdoMetDC activity has been described previously (Pegg & Pösö, 1983). Briefly, 250–300 μg of extract protein was incubated in 250 μl of reaction buffer containing 50 mM-sodium phosphate (pH 7.4), 2 mM-dithiothreitol, 3 mM-putrescine and 500 μM-[^{14}C]AdoMet (~ 1 μCi/μmol) for 15–20 min in a 37 °C shaking water bath. The reaction was terminated by the addition of an equal volume of 40% (w/v) trichloroacetic acid, and $^{14}CO_2$ was trapped on a methylbenzethonium hydroxide-soaked filter paper suspended in the sealed reaction vessel. Trapped $^{14}CO_2$ was quantified by liquid scintillation counting in Omnifluor cocktail (New England Nuclear). Proteins were determined by the method of Bradford (1976).

Plasma levels of MDL 73811 in mouse and rat plasma

Male Sprague–Dawley rats or CD1 mice were injected intraperitoneally with 50 mg of MDL 73811/kg and killed by asphyxiation with CO_2, and blood was collected via heart puncture. The blood cells were sedimented by centrifugation and 100% (w/v) trichloroacetic acid was added to the plasma to give a final trichloroacetic acid concentration of 10% (w/v). Precipitated proteins were removed by centrifugation and the supernatant was filtered through 0.22 μm-pore-size filters. MDL 73811 content was determined by h.p.l.c. analysis as described below.

Mammalian cells

L1210 mouse leukaemia 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. Rat hepatoma (HTC) cells were grown in spinner cultures as described previously (Hershko & Tomkins, 1966) in Earles minimum essential medium supplemented with 0.05% sodium bicarbonate, 0.9% Tricine, penicillin (12 μg/ml), streptomycin (12 μg/ml), 0.2% (w/v) glucose, 10% (v/v) horse serum and non-essential amino acids at 37 °C in air/CO_2 (19:1) and 90% humidity. Cells harvested for biochemical analysis were washed by centrifugation three times in a solution of 137 mM-NaCl, 3 mM-KCl, 16 mM-Na_2HPO_4, 0.2 mM-KH_2PO_4, $CaCl_2$ (0.2 mg/l) and $MgSO_4,7H_2O$ (0.2 mg/l). Cells to be analysed for AdoMetDC activity were suspended and sonicated in a solution of 111 mM-sodium phosphate (pH 7.4) containing 2 mM-dithiothreitol and 6.7 mM-putrescine. AdoMetDC activity in this extract was assayed as described for the trypanosome extract although with slightly different reaction conditions. Extract containing approx. 500 μg of protein was incubated for 1 h at 37 °C in a total volume of 0.6 ml of reaction buffer containing 111 mM-sodium phosphate (pH 7.4), 2 mM-dithiothreitol, 6.7 mM-putrescine and

[^{14}C]AdoMet. The L1210 cell AdoMetDC assays contained 200 μM-[^{14}C]AdoMet (1 μCi/μmol) and the HTC cell AdoMetDC assays contained 4 μM-[^{14}C]AdoMet (56.9 μCi/μmol).

Quantification of polyamines, AdoMet, dcAdoMet and MDL 73811

Trypanosomes were collected from individual rats, purified as described above, sedimented by centrifugation at 10000 *g* for 10 min and then extracted with 0.5 ml of 10% (w/v) trichloroacetic acid. L1210 and HTC cell pellets obtained as described above were suspended in 10% (w/v) trichloroacetic acid for h.p.l.c. analysis. Precipitated proteins were removed by centrifugation, dissolved in 1 ml of 1 M-NaOH and diluted 10-fold in 0.1 M-NaOH for measurement of protein content. The supernatant fractions were then filtered through 0.45 μm-pore-size membranes in preparation for h.p.l.c. analysis.

A reverse-phase chromatography system utilizing an h.p.l.c. apparatus from Beckman Instruments was used to separate putrescine and spermine. Samples were injected on to the system by a Waters 712 WISP autosampler. The sample was passed first through a Waters Bondapak C_{18} pre-column and then on to a Beckman Ultrasphere Ion Pairing 5 μm C_{18} (250 mm × 4.6 mm) column. Putrescine, spermidine and spermine were separated and quantified as described by Seiler & Knödgen (1985). Elution times were 26.4 min for putrescine, 34.4 min for spermidine and 37.6 min for spermine. Commercially available polyamine hydrochlorides were used as standards.

For separation of AdoMet and its metabolites in the absence of MDL 73811 the same h.p.l.c. apparatus and column was used, but a sodium phosphate buffer system (Wagner *et al.*, 1982) was employed to allow for greater sensitivity. Commercially available AdoMet, methylthioadenosine (MTA) and *S*-adenosyl-L-homocysteine (SAH), and dcAdoMet prepared as reported previously (Bitonti *et al.*, 1984), were used as standards. Elution times were: SAH, 26.4 min; AdoMet, 29.6 min; MTA, 33.2 min; dcAdoMet, 40.4 min. Alternatively, AdoMet, its metabolites, MDL 73811, and MDL 74281 were separated on the same h.p.l.c. apparatus using a sodium acetate buffer system which afforded less sensitivity than the sodium phosphate buffers, but which was necessary to separate MDL 73811 from dcAdoMet. The separation conditions were a modification of those used by Pegg *et al.* (1988). Buffer A contained 0.1 M-sodium acetate (pH 4.5) and 10.0 mM-octanesulphonate. Buffer B contained 0.154 M-sodium acetate (pH 4.5), 10 mM-octane sulphonate and 23% (v/v) acetonitrile. The gradient was started at 10% buffer B, went to 100% buffer B in 30 min, and then back to 10% buffer B in 2 min, after which the column was equilibrated for 10 min. The flow rate was 1 ml/min and the column temperature was 35 °C. Elution times were: SAH, 9.2 min; AdoMet, 14.8 min; MDL 74281, 15.2 min; MTA, 18.6 min; MDL 73811, 27 min; dcAdoMet, 27.8 min.

RESULTS

Administration of 50 mg of MDL 73811/kg to rats infected 3 days previously with *T. b. brucei* resulted in a rapid decrease in parasitaemia (Fig. 2*a*), with parasites becoming undetectable on microscopic observation of blood 5 h after a single injection (results not shown). A similar rapid decrease in parasitaemia was not observed in infected mice treated with 50 mg of MDL 73811/kg. However, the parasitaemia in these treated mice did not increase, even though the parasitaemias doubled in the untreated controls over the 5 h course of the experiment (Fig. 2*b*). The different responses to MDL 73811 by trypanosomes infecting rats and mice were not due to differences in the plasma levels or half-lives of the drug in these animals. The plasma levels (50–60 μM at 15 min) and half-lives (~ 10 min) of MDL 73811 in the plasma of rats and mice injected intraperitoneally with 50 mg of MDL 73811/kg were virtually identical (Fig. 3). The trypanocidal effect of MDL 73811 in rats was dose-dependent (Fig. 4),

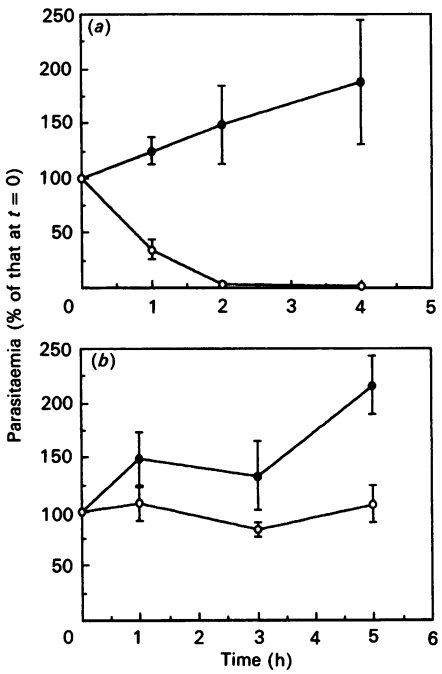

Fig. 2. Changes in the *T. b. brucei* parasitaemia of infected rats and mice following single intraperitoneal injections of MDL 73811

The parasitaemia of male Sprague–Dawley rats (*a*) or male CD-1 mice (*b*) infected with *T. b. brucei* was determined by collecting blood via tail cuts and counting the parasites on a haemocytometer. The animals were then injected intraperitoneally with 50 mg of MDL 73811/kg and the parasitaemia of individual animals was compared with the parasitaemia at $t = 0$. At 5 h after treatment trypanosomes were undetectable by microscopic observation of the blood of infected rats. The values represent means ± S.E.M. Parasitaemia did not increase significantly in treated mice ($P < 0.05$). ●, Untreated; ○, treated with MDL 73811.

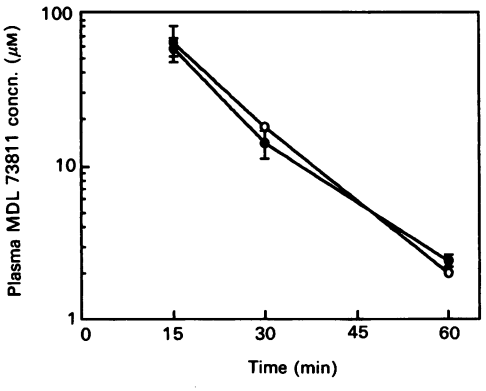

Fig. 3. Plasma concentrations of MDL 73811 in rat and mouse plasma after intraperitoneal injection of MDL 73811 (50 mg/kg)

Rats (○) and mice (●) were injected intraperitoneally with MDL 73811 (50 mg/kg) and killed at the indicated time points. Blood was collected via heart puncture, blood cells were sedimented by centrifugation and the plasma was analysed for MDL 73811 content by h.p.l.c. using the sodium acetate buffer system. The values represent the means ± S.D. for three animals.

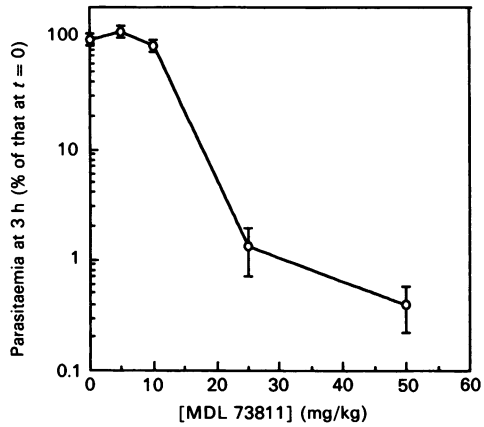

Fig. 4. The trypanocidal effect of MDL 73811 in rats is also a dose-dependent phenomenon

The parasitaemia of rats infected with *T. b. brucei* was determined by counting the numbers of parasites in blood collected by tail cuts. The rats were then injected intraperitoneally with MDL 73811 at the doses indicated. Blood was collected after 3 h and parasitaemia was determined. The values represent means ± S.E.M. from 3–6 rats.

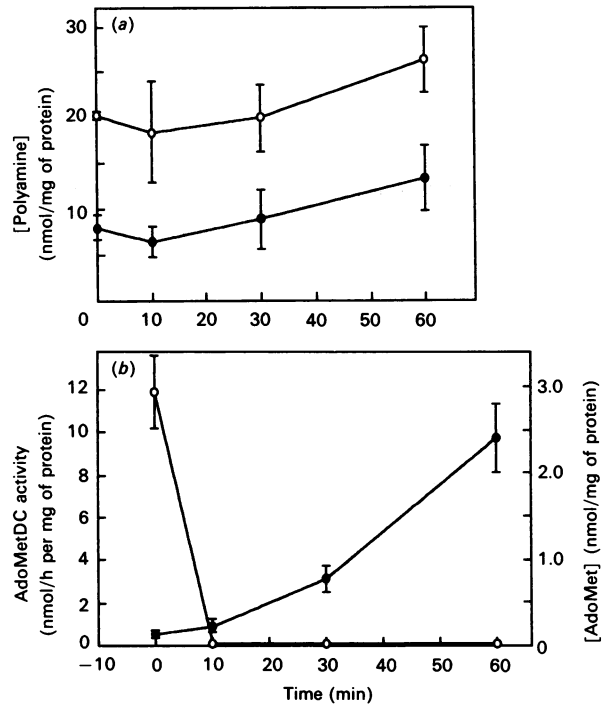

Fig. 5. Acute changes in polyamine levels, AdoMet levels and AdoMetDC activity in trypanosomes collected from infected rats treated with MDL 73811

Rats infected with *T. b. brucei* were injected intraperitoneally with MDL 73811 (25 mg/kg). The animals were killed at the indicated time points, blood was collected by heart puncture, and the trypanosomes were separated from blood by anion-exchange chromatography and were assayed for (*a*) polyamine levels (○, putrescine; ●, spermidine) and (*b*) AdoMet content (●) and AdoMetDC activity (○). AdoMetDC content was assayed by h.p.l.c. using the sodium phosphate buffer system. The values represent means ± S.D. for trypanosomes collected from 3–5 rats.

with the rapid disappearance of parasites from the blood being observed only in animals treated with at least 25 mg of MDL 73811/kg. Untreated control animals and those treated with

Table 1. Acute effects of 50 μM-MDL 73811 on L1210 and HTC cell AdoMet and polyamine levels and AdoMetDC activity

MDL 73811 (50 μM) was added to subconfluent L1210 and HTC cultures. At various times the cells were washed with phosphate-buffered saline and analysed by h.p.l.c. for AdoMet (using the sodium acetate buffers) and polyamine levels and for AdoMetDC activity. The L1210 values are the means ± S.E.M. for three culture flasks. The HTC values are from single culture flasks. N.D., not detected.

(*a*) L120 cells

Group	Levels (nmol/mg of protein) AdoMet	Putrescine	Spermidine	Spermine	AdoMetDC activity (pmol/h per mg of protein)
Control					
0 h	3.9±0.3	6.7±0.6	24.7±1.2	8.1±0.6	3.7±0.1
6 h	4.5±0.1	6.6±0.1	28.0±0.5	9.3±0.4	2.9±0.3
MDL 73811 (50 μM)					
0.5 h	4.2±0.2	5.7±0.2	22.9±1.1	6.5±0.8	N.D.
1 h	5.3±0.3	5.9±0.5	24.8±2.4	6.9±0.6	N.D.
2 h	5.8±0.6	7.9±1.0	23.7±2.5	7.4±1.0	N.D.
4 h	6.7±0.5	12.2±0.5	23.2±1.3	6.8±0.2	N.D.
6 h	5.9±0.3	16.0±2.0	19.3±1.1	6.0±0.2	N.D.

(*b*) HTC cells

Group	Levels (nmol/10^6 cells) AdoMet	Putrescine	Spermidine	Spermine	AdoMetDC activity (nmol/h per 10^9 cells)
Control					
0 h	0.55	1.3	1.8	2.7	2.2
6 h	0.57	0.7	1.8	2.3	1.6
MDL 73811 (50 μM)					
0.5 h	0.54	1.0	1.1	2.0	N.D.
1 h	0.73	1.1	1.3	2.2	N.D.
2 h	1.00	1.0	1.3	1.9	N.D.
3 h	0.83	1.1	1.3	1.8	N.D.
4 h	0.83	1.6	1.4	2.2	N.D.
6 h	0.90	2.2	1.4	1.8	N.D.

either 5 or 10 mg/kg of MDL/kg died within 24 h of drug administration, whereas those treated with 25 or 50 mg of MDL 73811/kg survived for an additional 10–12 days.

Putrescine and spermidine levels in trypanosomes collected from infected rats treated with MDL 73811 did not change significantly within the first 1 h after drug treatment (Fig. 5*a*), a time at which parasitaemia was markedly decreased. However, a 20-fold increase in parasite AdoMet was detected within this same time frame (Fig. 5*b*). Trypanosomal AdoMetDC activity was inhibited maximally in trypanosomes collected 10 min after treatment (Fig. 5*b*). Trypanosome dcAdoMet, which was very low in control trypanosomes (~ 0.01 nmol/mg of protein), was undetectable in treated parasites. SAH levels were not altered in trypanosomes within 1 h of injecting infected rats with 50 mg of MDL 73811/kg (control, 2.6±0.1 nmol/mg of protein; treated, 3.3±1.0 nmol/mg of protein). Treatment of *T. b. brucei*-infected rats with Berenil (2.5 mg/kg for 1 h) or MGBG (100 mg/kg for 3 h), both of which are known to inhibit trypanosomal AdoMetDC (Bitonti *et al.*, 1986), increased parasite AdoMet

Table 2. Eflornithine treatment of infected rats increases *T. b. brucei* AdoMet levels

Rats infected with *T. b. brucei* were treated with 4% eflornithine in drinking water for 36 h. The rats were killed, blood was collected by heart puncture and the trypanosomes were separated from the blood by anion-exchange chromatography. The trypanosomes were assayed for polyamines, dcAdoMet and AdoMet levels and for AdoMetDC activity. The sodium phosphate buffer system was used to assay AdoMet and dcAdoMet. The values are means ± S.E.M. in trypanosomes collected from five individual animals. N.D., not detected.

Treatment (36 h)	Putrescine (nmol/mg of protein)	Spermidine (nmol/mg of protein)	dcAdoMet (pmol/mg of protein)	AdoMet (nmol/mg of protein)	AdoMetDC activity (nmol/h per mg of protein)
Untreated	2.1 ± 0.1	13.3 ± 0.5	6.2 ± 0.4	0.78 ± 0.24	8.8 ± 0.6
4% Eflornithine	N.D.	5.2 ± 0.4	30000 ± 8000	56 ± 21	0.9 ± 0.3

Table 3. Antitrypanosomal activity of ODC inhibitors correlates with increases in AdoMet levels

Mice infected with *T. b. brucei* were treated with ODC inhibitors in their drinking water. After 36 h, the mice were killed, blood was collected by heart puncture and trypanosomes were separated from blood cells by ion-exchange chromatography. The trypanosomes were analysed for polyamine, AdoMet and dcAdoMet content. The sodium phosphate buffer system was used to assay for AdoMet and dcAdoMet. The values represent means ± S.E.M. of 7–10 mice. N.D., none detected. The putrescine, spermidine and AdoMet levels in trypanosomes from treated mice are all significantly different from those from controls ($P < 0.01$), except for the AdoMet levels in parasites from mice treated with Δ-MFMO-C_2H_5.

Treatment	Antitrypanosomal activity	Levels (nmol/mg of protein)			
		Putrescine	Spermidine	AdoMet	dcAdoMet
No treatment	–	6.0 ± 0.3	29.3 ± 1.1	1.1 ± 0.1	0.05 ± 0.01
2% Eflornithine	Yes	N.D.	12.6 ± 2.1	21.7 ± 4.7	12.5 ± 4.9
0.5% Δ-MFMO-CH_3	Yes	N.D.	7.3 ± 2.0	26.8 ± 6.9	14.3 ± 5.9
0.5% Δ-MFMO-C_2H_5 ester	No	N.D.	3.1 ± 0.6	3.2 ± 0.8	1.6 ± 0.4

levels from 0.23 ± 0.03 to 1.67 ± 0.14 and 1.52 ± 0.36 nmol/mg of protein respectively.

The effect of MDL 73811 treatment on AdoMet levels appears to be specific for the trypanosome. Unlike AdoMet levels in trypanosomes treated with MDL 73811, mammalian cell AdoMet levels did not increase dramatically with MDL 73811 exposure (Table 1). Only a 1.5–2-fold increase in AdoMet was observed in cultured L1210 and HTC cells after a 6 h exposure to 50 μM-MDL 73811 (approximately the plasma concentration of MDL 73811 in rats and mice 15 min after injection with 50 mg/kg). AdoMetDC activity was undetectable within 30 min of the addition of MDL 73811 to the cultures, which is consistent with the rapid inhibition of this enzyme observed in trypanosomes. This inhibition resulted in an increase in putrescine content (approx. 2.5-fold at 6 h), although spermidine and spermine levels were unchanged over the course of this experiment.

Trypanosomes from *T. b. brucei*-infected rats treated with eflornithine were found to be depleted of putrescine and to have spermidine levels decreased by 60%, but they also had elevated dcAdoMet (> 4000-fold) and AdoMet (up to 50-fold) levels (Table 2), confirming the original findings of Yarlett & Bacchi (1988). Treatment of *T. b. brucei*-infected mice with the ODC inhibitors eflornithine, Δ-MFMO-CH_3 (MDL 72403) or Δ-MFMO-C_2H_5 (MDL 72430) resulted in depletion of putrescine and decreased spermidine levels in the parasites (Table 3). Eflornithine and Δ-MFMO-CH_3, which cure murine *T. b. brucei* infections (Bitonti *et al.*, 1985), also elevated parasite AdoMet levels, but Δ-MFMO-C_2H_5, which does not cure this murine trypanosome infection (Bitonti *et al.*, 1985), did not elevate parasite AdoMet levels (Table 3). Levels of dcAdoMet were relatively higher in trypanosomes from mice treated with Δ-MFMO-CH_3 or eflornithine compared with those in trypanosomes from mice treated with Δ-MFMO-C_2H_5.

DISCUSSION

Trypanosome AdoMetDC activity was inhibited completely within 10 min after injection of MDL 73811. Based on a trypanosome cell volume of 58 μl/10^9 trypanosomes (Opperdoes *et al.*, 1984), the concentration of MDL 73811 in the trypanosomes was calculated to be 1.9 ± 0.1 mM. Kinetic data for the inhibition of trypanosome AdoMetDC by MDL 73811 *in vitro* (Bitonti *et al.*, 1990) indicated that the half-life for the inhibition of AdoMetDC with 2 mM-MDL 73811 would be approx. 0.3 min, accounting for the absence of AdoMetDC activity in the trypanosomes at 10 min. The high intracellular concentration of MDL 73811 also suggested that trypanosomes have an active uptake system for which MDL 73811 is a substrate as the concentration of MDL 73811 in rat plasma 15 min after intraperitoneal injection of 50 mg of MDL 73811/kg was only 50–60 μM. Because trypanosomes cannot synthesize purines (reviewed in Hammond & Gutteridge, 1984) they are dependent on exogenous purine sources and therefore have a specific active transport system for purine nucleosides (Hammond & Gutteridge, 1984; Gottlieb & Dwyer, 1988). It is possible that MDL 73811, an adenosine analogue, may be a substrate for this system. Because the half-life of MDL 73811 in mouse and rat blood is less than 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*.

Subsequent to the inhibition of AdoMetDC activity in the trypanosomes a rapid and large increase in levels of AdoMet occurred. The rapid build-up of AdoMet on inhibition of AdoMetDC suggests that a majority of the AdoMet synthesized by trypanosomes is normally utilized in the synthesis of spermidine. Rapid changes in AdoMet levels in the absence of significant changes in polyamine levels, over a time period in

which MDL 73811 reduces the parasitaemia of treated rats, suggests that the elevation of AdoMet may be trypanocidal. Similar changes in AdoMet did not occur in cultured mammalian cells and may explain why MDL 73811 selectively kills the parasites while having no apparent toxic effects on the mammalian host. A possible explanation for these data is that mammalian AdoMet synthetase is known to be regulated by AdoMet (Oden *et al.*, 1983; Geller *et al.*, 1986), whereas the trypanosome enzyme may be poorly regulated.

Further evidence suggesting that trypanosomes may be sensitive to increased intracellular AdoMet levels was obtained in experiments with trypanosomes taken from mice treated with inhibitors of putrescine biosynthesis. Treatment of *T. b. brucei*-infected rats with eflornithine paradoxically decreased AdoMetDC activity and elevated AdoMet levels in trypanosomes taken from treated rats, and these changes were accompanied by decreased polyamine levels and increased dcAdoMet, confirming the findings of Yarlett & Bacchi (1988). Although depletion of polyamines and increased dcAdoMet levels have been reported previously for both trypanosomes (Bacchi *et al.*, 1983; Fairlamb *et al.*, 1987) and mammalian cells (Mamont *et al.*, 1978, 1981, 1982) treated with ODC inhibitors, the increased AdoMet levels and the decreased AdoMetDC activity are apparently unique to the trypanosome. Elevated AdoMet levels in eflornithine-treated mammalian cells has not ben observed, presumably because AdoMetDC activity is induced in mammalian cells in which polyamines have been depleted (Mamont *et al.*, 1981, 1982; Pegg *et al.*, 1982). The elevation of trypanosomal AdoMet levels following eflornithine treatment may be due to the 4000–7000-fold increase in trypanosome dcAdoMet, which is significantly greater than the 500-fold increase in dcAdoMet reported in mammalian cells treated with eflornithine (Pegg & McCann, 1982; Pegg, 1984). Despite the lowered AdoMetDC activity in the trypanosomes after 36 h of eflornithine treatment, the elevation of dcAdoMet indicates that AdoMetDC remained active, possibly induced by the depletion of putrescine after ODC inhibition, earlier in the treatment time. Formation of dcAdoMet in the absence of putrescine, a substrate necessary for the metabolism of dcAdoMet, resulted in a concentration of dcAdoMet in the trypanosome (3.5 ± 0.9 mM at 36 h) which may be high enough to competitively inhibit AdoMetDC or even allow dcAdoMet to act as an irreversible inhibitor of AdoMetDC. Yeast (Pösö *et al.*, 1975) and mammalian (Yamanoha & Samejima, 1980) AdoMetDCs are competitively inhibited by dcAdoMet, with K_i values of 1 μM and 6.3 μM respectively, and Kolb *et al.* (1982) reported the time-dependent inhibition of mammalian AdoMetDC by 1 mM-dcAdoMet. With AdoMetDC inhibited, increases in AdoMet would occur similar to those seen in trypanosomes treated with MDL 73811. Thus it is possible that trypanosome-specific increases in AdoMet following depletion of putrescine may be an important mechanism by which ODC inhibitors exert their antitrypanosomal effect. This is supported by the finding that Δ-MFMO-C_2H_5, which does not cure murine *T. b. brucei* infections, depleted trypanosome polyamines but did not significantly alter trypanosome AdoMet levels, whereas Δ-MFMO-CH_3 and eflornithine, which cure these murine infections, depleted polyamines and increased AdoMet levels. The relatively higher dcAdoMet levels in trypanosomes treated with Δ-MFMO-CH_3 or eflornithine compared with the dcAdoMet level in trypanosomes treated with Δ-MFMO-C_2H_5 suggests that high levels of dcAdoMet may inhibit AdoMetDC and be responsible for the build-up of AdoMet in trypanosomes.

The mechanism by which increases in AdoMet levels may result in trypanosome cell death is not known, but one possibility is via aberrant methylation. Methylation reactions in which AdoMet is the methyl donor are regulated in part by the ratio of AdoMet to SAH (Ueland, 1982), the metabolite of AdoMet formed by methyl donation. Since SAH levels did not increase significantly in trypanosomes treated with MDL 73811, it is possible that the elevated AdoMet/SAH ratio results in improper or hypermethylation. Penketh *et al.* (1990) reported that methylating agents cured murine infections of *T. b. rhodesiense* and that the activities of these compounds appeared to be related to their methylating action, as ethylating agents were not trypanocidal. The finding that MDL 73811 appears to be acutely trypanostatic in mice, rather than rapidly trypanocidal as in rats, suggests that changes in the trypanosome after MDL 73811 treatment may also make the parasite more susceptible to serum factors.

In conclusion, treatment of infected rats with MDL 73811 results in a rapid elevation of *T. b. brucei* AdoMet levels which correlates temporally with the antitrypanosomal action of this drug. It is possible that this specific elevation of AdoMet in trypanosomes may be responsible for the specific antitrypanosomal action of MDL 73811 as well as of other antitrypanosomal drugs that inhibit AdoMetDC. It also was shown that inhibition of ODC indirectly lowers AdoMetDC activity in trypanosomes and results in the specific elevation of AdoMet levels in the parasite. We propose that this elevation in trypanosomal AdoMet levels may be responsible for the specific antitrypanosomal effects of ODC inhibitors such as eflornithine and Δ-MFMO-CH_3.

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