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Characterization of *Trypanosoma brucei brucei* *S*-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanylhydrazone)

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Trypanosoma brucei brucei *S*-adenosyl-L-methionine (AdoMet) decarboxylase was found to be relatively insensitive to activation by putrescine as compared with the mammalian enzyme, being stimulated by only 50% over a 10000-fold range of putrescine concentrations. The enzyme was not stimulated by up to 10 mM-Mg^{2+}. The K_m for AdoMet was 30 μM, similar to that of other eukaryotic AdoMet decarboxylases. *T. b. brucei* AdoMet decarboxylase activity was apparently irreversibly inhibited *in vitro* by Berenil and reversibly by pentamidine and methylglyoxal bis(guanylhydrazone). Berenil also inhibited trypanosomal AdoMet decarboxylase by 70% within 4 h after administration to infected rats and markedly increased the concentration of putrescine in trypanosomes that were exposed to the drug *in vivo*. Spermidine and spermine blocked the curative effect of Berenil on model mouse *T. b. brucei* infections. This effect of the polyamines was probably not due to reversal of Berenil's inhibitory effects on the AdoMet decarboxylase.

INTRODUCTION

S-Adenosyl-L-methionine (AdoMet) decarboxylase (EC 4.1.1.50) is an obligatory enzyme for the biosynthesis of the polyamines spermidine and spermine in higher eukaryotes (Pegg & McCann, 1982) and also in eukaryotic protozoa such as *Trypanosoma brucei brucei* (Bacchi, 1981; Bacchi *et al.*, 1979; Dave *et al.*, 1976). AdoMet decarboxylase has been considered as a potential target for chemotherapy of proliferative disorders such as cancer since Williams-Ashman & Schenone (1972) demonstrated that methylglyoxal bis(guanylhydrazone) (MGBG), a cytotoxic drug which inhibits cell proliferation (Kay & Pegg, 1973), was also a potent competitive inhibitor of AdoMet decarboxylase activity. MGBG, as well as numerous of its analogues, possess significant palliative as well as curative properties against mouse *Trypanosoma brucei* infections (Jaffe, 1965; Risby, 1976; Ulrich *et al.*, 1982; Bacchi *et al.*, 1983; Ulrich & Cerami, 1984). Furthermore, it was demonstrated that two other trypanocidal agents, Berenil (4,4′-diaminidinodiazoaminobenzene; diminazene aceturate) and pentamidine [*p*,*p*′-(pentamethylenedioxy)dibenzamidine; pentamidine isethionate], irreversibly inhibit AdoMet decarboxylase activity from rat liver, yeast and *Escherichia coli* (Karvonen *et al.*, 1985), and it was suggested that inhibition of this enzyme might play a role in the therapeutic efficacy of the compounds for the treatment of protozoal infections. The latter hypothesis is attractive because of the demonstrated efficacy of α-difluoromethylornithine, an enzyme-activated inhibitor of ornithine decarboxylase, the first enzyme in the polyamine-biosynthetic pathway (Metcalf *et al.*, 1978; Bitonti *et al.*, 1985), for the treatment of experimental mouse (Bacchi *et al.*, 1980) and naturally occurring human (Sjoerdsma & Schechter, 1984; Sjoerdsma *et al.*, 1984; Van Nieuwenhove *et al.*, 1985) trypanosome infections.

In the present study we have partially characterized AdoMet decarboxylase from *T. b. brucei* and examined the effects of MGBG, Berenil and pentamidine on this protozoal enzyme. Berenil apparently irreversibly inhibited trypanosomal AdoMet decarboxylase both *in vitro* and *in vivo*, whereas pentamidine and MGBG were found to be reversible inhibitors of the enzyme.

EXPERIMENTAL

Organisms

Trypanosoma brucei brucei (isolate EATRO 110) were maintained in the laboratory by syringe passage in 300 g male Sprague–Dawley rats. Trypanosomes were harvested from 72 h-infected rats as described by Bitonti *et al.* (1985) and purified by DEAE-cellulose chromatography (Lanham & Godfrey, 1970).

Preparation of cell extracts and enzyme assays

Trypanosomes were disrupted by sonication (Branson Cell Disruptor 350, setting 1; 3 × 30 s) in 5 mM-sodium phosphate (pH 7.4) containing 0.1 mM-EDTA and 2 mM-dithiothreitol. Sonicated cells were then centrifuged at 12000 rev./min (Sorvall SS34 rotor) for 30 min to remove cellular debris. The supernatant, containing 8–15 mg of protein/ml, was stored frozen at −20 °C without loss of activity for up to 1 month. AdoMet was assayed as described by Pegg & Pösö (1983). The enzyme reaction contained 125 mM-sodium phosphate (pH 7.4), 3 mM-dithiothreitol, 0.5 mM-AdoMet, 0.2 μCi of *S*-adenosyl-L-[*carboxy*-^{14}C]methionine (Ado[^{14}C]Met), 3 mM-putrescine and usually 0.5 mg of protein. Protein concentrations were measured by the method of Lowry *et al.* (1951). For experiments with pentamidine, Tris/HCl was substituted for sodium phosphate in both the disruption and assay buffers in an attempt to avoid precipitation

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; MGBG, methylglyoxal bis(guanylhydrazone) {1,1′-L-[(methylethanediylidine)dinitrilo]diguanidine}.

of the drug at high concentration. Since 10 mM-pentamidine precipitated even in Tris/HCl, we tried selectively removing the precipitated material by centrifugation, but this resulted in a noticeable loss of enzyme activity, probably owing to sedimentation of the enzyme along with the drug.

Inhibition of AdoMet decarboxylase activity

To test the reversibility of the inhibition of the enzyme with Berenil, pentamidine and MGBG, a sample of the enzyme preparation was incubated for 30 min at 20 °C in the presence of an inhibitory concentration of drug. A sample of the inhibited enzyme was assayed for residual activity, and the rest of the inhibited enzyme was applied to a 10 ml Sephadex G-25 column (PD-10 prepacked columns; Pharmacia). The column was washed with 2.5 ml of disruption buffer (above), and then AdoMet decarboxylase activity was eluted with an additional 1.5 ml of buffer, and activity was determined as described above. Enzyme activity was decreased by approx. 25% during the incubation at 20 °C in the absence of inhibitors.

Measurement of intracellular polyamines

Trypanosomes were harvested and purified as described above. After the purified trypanosomes were washed twice in 90 mM-Tris/HCl (pH 7.8) containing 50 mM-NaCl and 2% (w/v) glucose, the organisms were sedimented by centrifugation at 15000 *g* for 10 min and then extracted with small volumes of 0.4 M-$HClO_4$ in preparation for polyamine analysis. Precipitated proteins were removed by centrifugation and filtration on 0.45 μm filters, and polyamines were determined by h.p.l.c. exactly as described by Wagner *et al.* (1982).

All experiments presented herein were repeated two or three times with similar results.

Chemicals

AdoMet was purchased from Boehringer–Mannheim, Ado[^{14}C]Met (62 Ci/mmol) from New England Nuclear, and Berenil and MGBG were from Sigma. Pentamidine was generously given by May and Baker.

RESULTS

AdoMet decarboxylase from *Trypanosoma brucei brucei* was found to have a K_m for AdoMet of 30 μM and was weakly stimulated (50%) by putrescine (Fig. 1). The trypanocidal drugs Berenil, pentamidine and MGBG inhibited AdoMet decarboxylase at concentrations as low as 10 μM (Fig. 2). Complete inhibition of the enzyme with MGBG and Berenil was obtained at 1 mM drug, whereas 10 mM-pentamidine was necessary for complete inhibition. The inhibition of the enzyme by the three trypanocides was competitive in nature, and apparent K_i values for the drugs were found to be 20 μM for Berenil, 32 μM for MGBG and 50 mM for pentamidine.

Berenil (1 mM) apparently irreversibly inhibited AdoMet decarboxylase, since chromatography of the inhibited enzyme on Sephadex G-25 failed to restore enzyme activity (Table 1). The inhibition by both MGBG and pentamidine was reversed by chromatography on Sephadex G-25. Chromatography of the enzyme, which had been inhibited with MGBG or pentamidine (not centrifuged) on Sephadex G-25, resulted in AdoMet decarboxylase activity which was higher than the

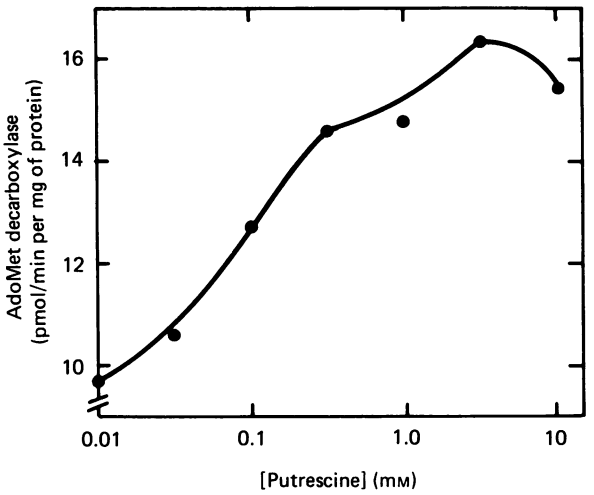

Fig. 1 Putrescine stimulation of *T. b. brucei* AdoMet decarboxylase activity

AdoMet decarboxylase activity was measured in the presence of various putrescine concentrations as described in the Experimental section.

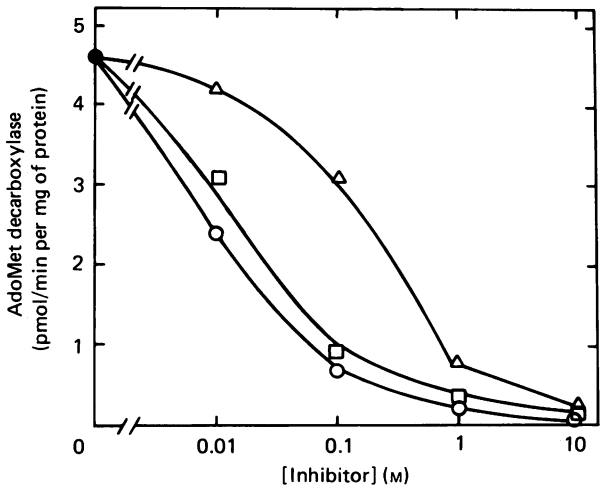

Fig. 2. Inhibition of *T. b. brucei* AdoMet decarboxylase activity by MGBG, pentamidine and Berenil

AdoMet decarboxylase was measured as described in the Experimental section in the absence or presence of various concentrations of MGBG (○), pentamidine (△) or Berenil (□).

corresponding controls (Table 1). Although we have no ready explanation for this phenomenon, it could be due to the drugs' effect on the elution of selected proteins from the Sephadex column in the AdoMet decarboxylase preparations, which resulted in a partial purification of the enzyme.

Berenil was also found to inhibit effectively the enzyme *in vivo* (Table 2). At 4 h after an intraperitoneal dose of 2.5 mg of Berenil/kg was given to infected rats, the enzyme activity was decreased by 70% in trypanosomes harvested from the treated animals. The intracellular concentration of putrescine was significantly increased to greater than 5-fold control values after inhibition of

Table 1. Reversibility of the inhibition of AdoMet decarboxylase by Berenil, pentamidine and MGBG

T. b. brucei extract was incubated in the absence or presence of 1 mM-Berenil or -MGBG (Expt. 1) or 10 mM-pentamidine (Expt. 2) for 30 min at 20 °C. After incubation with the drugs, the enzyme preparation was applied to a Sephadex G-25 column and eluted and assayed as described in the Experimental section. In Expt. 2, pentamidine formed a noticeable precipitate on addition to the enzyme preparation. Either the enzyme preparation was added to Sephadex G-25 without prior centrifugation or the preparation was centrifuged (2500 rev./min, 10 min) and the resulting supernatants were assayed both before and after chromatography on Sephadex G-25.

	AdoMet decarboxylase activity (pmol of CO_2/min per mg of protein)	
	Before Sephadex G-25	After Sephadex G-25
Expt. 1		
Control	3.1	3.6
Berenil	0.84	1.2
MGBG	0.75	4.8
Expt. 2		
Control	7.4	7.7
Pentamidine, not centrifuged	1.8	11.8
Pentamidine, centrifuged	0.68	3.2

AdoMet decarboxylase activity by Berenil. Intracellular spermidine also showed a small but statistically significant increase after Berenil treatment (Table 2).

The polyamines putrescine, spermidine and spermine prevented or partially prevented the curative effect of Berenil on a *T. b. brucei* infection in mice (Table 3). Injection of the polyamines along with Berenil resulted in a greater mortality rate than when Berenil was given alone, although the animals treated with both the drug and putrescine or spermidine survived longer than control animals. However, spermidine and spermine did not block the inhibition of AdoMet decarboxylase by Berenil *in vitro* and were themselves inhibitory at 10 mM (Table 4).

Table 3. Reversal of Berenil-induced cures of trypanosomiasis in mice by polyamines

Mice were infected with 2.5×10^5 trypanosomes. Drug treatments began 24 h after infection. Berenil was given intraperitoneally at a dose of 2.5 mg/kg, putrescine at a dose of 500 mg/kg, spermidine at a dose of 100 mg/kg and spermine at a dose of 40 mg/kg.

Treatment	No. of mice surviving after 60 days	Period of survival for mice that died (days)
None	0/6	4, 4, 4, 4, 5, 6
Berenil	5/5	(none died)
Berenil + putrescine	4/5	30
Berenil + spermidine	3/5	11, 14
Berenil + spermine	2/5	5, 6, 6

Table 4. Effects of spermidine, spermine and berenil on *T. b. brucei* AdoMet decarboxylase

AdoMet decarboxylase activity was assayed in the presence of the indicated concentrations of spermidine with or without 1 mM-Berenil as described in the Experimental section.

Additions	AdoMet decarboxylase activity (pmol of CO_2/min per mg of protein)	
	Without Berenil	With Berenil
	2.65	0.13
Spermidine (mM)		
0.01	2.83	0.21
0.1	2.82	0.18
1.0	2.82	0.18
10	0.50	0.13
Spermine (mM)		
0.01	2.78	0.16
0.1	3.02	0.18
1.0	2.58	0.17
10	0.65	0.11

Table 2. Effects of Berenil on *T. b. brucei* AdoMet decarboxylase activity and intracellular polyamine concentrations

Rats were infected with 4×10^7 trypanosomes and the infections were allowed to develop for 48 h. Animals were then injected intraperitoneally with Berenil (2.5 mg/kg) and trypanosomes were harvested from blood 4 h later. In one experiment trypanosomes were processed for determination of AdoMet decarboxylase activity, whereas in a second experiment the trypansomes were extracted with 0.4 M-$HClO_4$ for polyamine analysis. Values represent means ± S.D. for the numbers of determinations shown in parentheses: *significantly different from control (Student t test; $P < 0.005$).

	AdoMet decarboxylase activity (pmol of CO_2/min per mg of protein)	Polyamine concn. (nmol/10^9 trypanosomes)	
		Putrescine	Spermidine
Control	9.2 ± 1.3 (3)	49 ± 9 (5)	209 ± 45 (5)
Berenil	2.9 ± 0.7 (4)	284* ± 24 (3)	316* ± 10 (3)

DISCUSSION

T. b. brucei had a K_m for AdoMet of 30 μM, which is similar to the K_m obtained with the well-characterized AdoMet decarboxylases from mammalian, yeast and bacterial sources (Pegg & Jacobs, 1983). The trypanosomal enzyme was stimulated by only 50% over a 10000-fold range of putrescine concentration, similar to the putrescine-insensitive AdoMet decarboxylase from *Physarum polycephalum* (Mitchell & Rusch, 1973) and *Tetrahymena pyriformis* (Pösö *et al.*, 1975) and unlike AdoMet decarboxylase from mammalian or yeast sources (Pösö *et al.*, 1976), which are stimulated several-fold by putrescine. Furthermore, unlike the bacterial enzyme (Pösö *et al.*, 1976), which is highly stimulated by Mg^{2+}, trypanosomal AdoMet decarboxylase was totally unaffected by Mg^{2+} up to 10 mM (A. J. Bitonti, unpublished work). The enzyme was also not stimulated by low concentrations of spermidine or spermine and was inhibited by higher concentrations of the two polyamines.

Berenil, MGBG and pentamidine all inhibited trypanosomal AdoMet decarboxylase. Berenil was apparently a potent irreversible inhibitor both *in vitro* and *in vivo*, which is in agreement with the results of Karvonen *et al.* (1985) obtained with rat liver, yeast and bacterial AdoMet decarboxylase. However, we could not demonstrate irreversible inhibition of the enzyme with pentamidine. Inhibition by the latter compound was readily reversed on chromatography on Sephadex G-25, as was the inhibition by MGBG, known to be a reversible competitive inhibitor of AdoMet decarboxylase (Williams-Ashman & Schenone, 1972). At present we have no explanation for this discrepancy. The inhibition of the *T. b. brucei* enzyme by MGBG was similar to the inhibition by this compound of other putrescine-insensitive AdoMet decarboxylase activities from *Physarum polycephalum* or *E. coli* (Pegg & Jacobs, 1983). The K_i of 32 μM for MGBG against the *T. b. brucei* enzyme is markedly higher than the K_i of 0.3 μM determined previously for the putrescine-sensitive AdoMet decarboxylase from rat prostrate (Pegg & Jacobs, 1983). We have also noted that Berenil, pentamidine and MGBG were not entirely specific for AdoMet decarboxylase, but were inhibitory for *T. b. brucei* ornithine decarboxylase activity at concentrations 10–100-fold higher than those needed for similar inhibition of AdoMet decarboxylase (A. J. Bitonti, unpublished work). The relatively weak inhibition of ornithine decarboxylase by Berenil is probably not of pharmacological importance, since Berenil increased trypanosomal putrescine concentration *in vivo*, suggesting that AdoMet decarboxylase was primarily affected.

The question of whether or not the inhibition of AdoMet decarboxylase by Berenil, pentamidine and MGBG is related to their trypanocidal effects remains unanswered. It is noteworthy that, 4 h after injection, Berenil inhibited *T. b. brucei* AdoMet decarboxylase *in vivo* and markedly increased the intracellular putrescine concentration, as would be expected when the enzyme was blocked. There was also a small but statistically significant rise in intracellular spermidine concentration 4 h after Berenil administration. This increase was probably a result of the massive build-up of putrescine coupled with an incomplete block of AdoMet decarboxylase activity by Berenil. This combination of events would allow more spermidine to be synthesized from putrescine and suggests that the intracellular concentration of putrescine rather than the intracellular concentration of decarboxylated AdoMet limits spermidine biosynthesis in *T. b. brucei*. It is unlikely that the increase in spermidine was due to increased degradation, since we have been unable to detect either spermidine acetylase or polyamine oxidase, the two enzymes involved in the degradation of spermidine in other eukaryotic cells, in *T. b. brucei* (A. J. Bitonti, unpublished work). Furthermore, we have demonstrated that spermidine is not rapidly depleted in *T. b. brucei* after inhibition of putrescine biosynthesis with α-difluoromethylornithine. Depletion of spermidine in trypanosomes treated with α-difluoromethylornithine appears to result from dilution owing to cell doubling rather than degradation of the polyamine (Giffin *et al.*, 1986). We are quite certain that the increased putrescine was not due to increased formation from spermidine, since, as stated above, we have been unable to detect polyamine acetylase or polyamine oxidase activity in *T. b. brucei*. However, we cannot absolutely rule out the possibility that Berenil does not inhibit putrescine degradation, although there is no evidence available to suggest that putrescine is metabolized in any way other than to spermidine by *T. b. brucei*. We were unable to determine if there was an eventual Berenil-induced depletion of spermidine, since Berenil kills the trypanosomes quite rapidly and sufficient organisms for polyamine analysis could not be collected at time points much in excess of 4 h. The effects of MGBG or pentamidine on polyamines were not investigated.

Certainly, Berenil does not work solely through polyamine metabolism, since it has been shown to block selectively kinetoplast DNA synthesis (Newton & LePage, 1967), but inhibition of AdoMet decarboxylase might contribute to the compound's overall efficacy as a trypanocide. At the very least, we can say that polyamines are somehow involved in the cytotoxicity of Berenil, pentamidine and similar compounds, since spermidine or spermine can block the curative effects of these compounds *in vivo* (Bacchi *et al.*, 1983; the present work). It was suggested by Bacchi (1981) that these trypanocidal agents might in fact act by displacing intracellular polyamines. It may be that these compounds also act by limiting the synthesis of spermidine via inhibition of AdoMet decarboxylase, but clearly the reversal of Berenil's effects is not due to the polyamines simply interfering with the binding of Berenil to the enzyme. It was possible that the polyamines reversed the effects of Berenil by inhibiting the uptake of the drug by the trypanosome. In studies *in vitro* designed to test for competition for uptake by *T. b. brucei* between Berenil and the polyamines, we have found that 2 μM-Berenil, a concentration that could be reasonably expected in serum after administration of 2.5 mg of Berenil/kg (Gilbert, 1983), did indeed partially limit the uptake of ^{14}C-labelled putrescine, spermidine and spermine by up to 40% in the case of putrescine and less in the cases of spermidine or spermine (results not shown). We do not consider that this competition for uptake is sufficient to account completely for the reversal of Berenil's effects *in vivo*. The trypanocidal effects of MGBG could not be blocked by co-administration of spermidine or spermine (Jaffe, 1965; Chang *et al.*, 1978), so the relationship of this drug's trypanocidal effects to

polyamine metabolism is tenuous. Further studies are needed to elucidate more fully the role of inhibition of AdoMet decarboxylase by Berenil in the trypanocidal effect of the drug and the mechanism by which the inhibition occurs.

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