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Effects of Intermediates of Methionine Metabolism and Nucleoside Analogs on *S*-Adenosylmethionine Transport by *Trypanosoma brucei brucei* and a Drug-resistant *Trypanosoma brucei rhodesiense*

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ABSTRACT. The effects of purine nucleoside analogs, polyamines, and established trypanocidal agents on the uptake of [8-^{14}C]adenosine and *S*-[*methyl*-^{3}H]adenosylmethionine (AdoMet) by bloodform trypanosomes of drug-susceptible *Trypanosoma brucei brucei* and a drug-resistant *Trypanosoma brucei rhodesiense* clinical isolate were compared. AdoMet uptake was not antagonized by ornithine or methionine (500 μM), adenosine (100 μM), or other purine nucleosides, including methylthioadenosine (MTA) at 500 μM. Hydroxyethylthioadenosine (HETA), a trypanocidal analog of methylthioadenosine, and sinefungin, an analog of AdoMet, were competitive with AdoMet transport in both isolates. Dipyridamole, an antagonist of the adenosine P_2 transporter, also competed with AdoMet transport in both isolates. The trypanocidal diamidines pentamidine, Berenil, CGP 40215, and the decarboxylated *S*-adenosylmethionine (dAdoMet) analog MDL 73811 (5′-{[(Z)-4-amino-2-butenyl]}methyl- amino}-5′-deoxyadenosine) competed with P_2 adenosine transport but did not inhibit AdoMet transport at 100 μM. Methylglyoxalbis(guanylhydrazone) (MGBG), an analog of dAdoMet, was a strong competitive inhibitor of adenosine transport at 100 μM, but did not inhibit AdoMet transport. The polyamines putrescine, spermine, and spermidine (1 mM) were examined for competition with adenosine and AdoMet transport. Putrescine significantly inhibited P_2 adenosine transport in both strains (in the presence of saturating inosine), but AdoMet transport was not affected by these polyamines. P_2 adenosine transport in both strains was highly inhibited by melarsen oxide and melamine, its key organic component, whereas AdoMet uptake was not affected by these agents. These findings further characterize distinguishing features of the unique AdoMet transporter in African trypanosomes, and indicate that the P_2 adenosine transporter remains functional in melarsen- and diamidine-resistant clinical isolates. BIOCHEM PHARMACOL **56**;1:95–103, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *S*-adenosylmethionine metabolism; methionine metabolism; methylthioadenosine; trypanocidal agents

African trypanosomes of the *Trypanosoma brucei* subgroup are parasitic extracellular flagellates found in the connective tissues of humans and livestock. AdoMet** (Fig. 1) is the source of the aminopropyl moiety for polyamine synthesis and the methyl donor for protein and lipid methylation [1, 2]. Studies of AdoMet metabolism in *T. brucei brucei* showed that conversion of [^{35}S]methionine to AdoMet occurs rapidly [3] and, under the stress of inhibitors of polyamine synthesis [4], AdoMet levels increase 20- to 50-fold [5, 6]. In a study of methionine metabolism, 90% of the exogenous methionine taken up was converted to AdoMet; however, AdoMet synthesized from methionine represented only about 10% of the pool, while transport of exogenous AdoMet accounted for 90% of the pool ([4]; B. Goldberg, unpublished results). Protein methylation accounted for approximately 20% of the methionine taken up and converted to AdoMet [2]. Most of the total methionine utilized by trypanosomes for protein methylation appeared as carboxymethylation, and approximately 27% of the total methionine taken up was utilized in phospholipid methylation [2].

In initial studies with *T. b. brucei*, we reported that AdoMet entered through a transporter that was competi-

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** *Abbreviations:* AdoMet, *S*-adenosylmethionine; CGP 40215, bicyclic analog of MGBG (cpd 11; see Ref. 28); DFMO, DL-α-difluoromethylomithine; HETA, hydroxyethylthioadenosine; KMTB, ketomethylthiobutyrate; MDL 73811, 5′-{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine; MGBG, methylglyoxalbis(guanylhydrazone); and MTA, methylthioadenosine.

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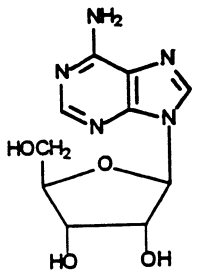
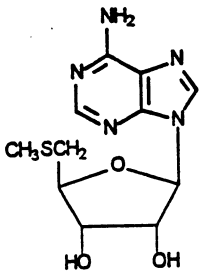


Adenosine



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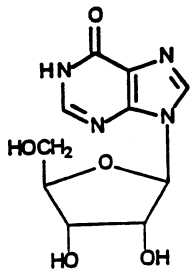

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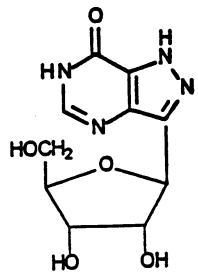

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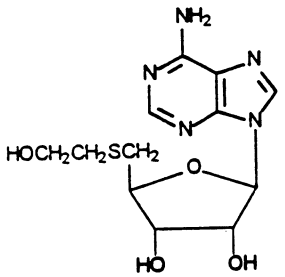

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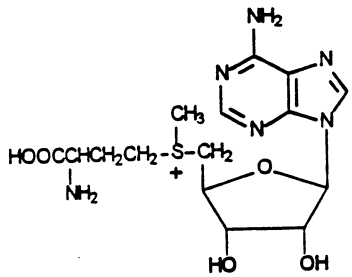
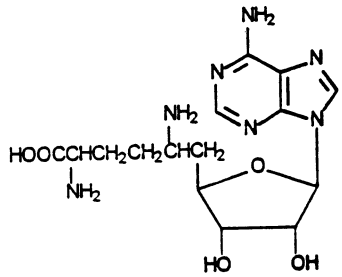
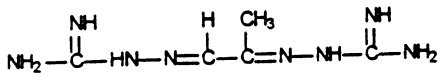


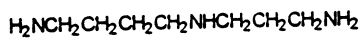
S-Adenosylmethionine



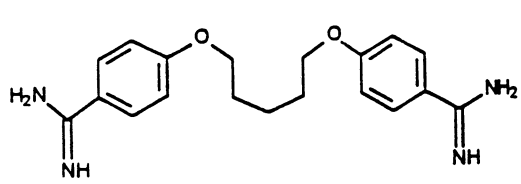
Sinefungin



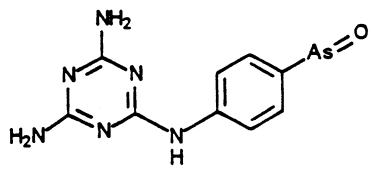
MGBG



Spermidine



Pentamidine



Melarsen oxide

FIG. 1. Structures of nucleosides and antagonists of nucleoside uptake.

tive with sinefungin and dipyridamole but not with ornithine, methionine, or adenosine [7]. Uptake by this transporter appears to be a significant physiological feature, since comparison of the pool size of [^{35}S]AdoMet formed from [^{35}S]methionine versus that from uptake of exogenous [*methyl*-^{3}H]AdoMet indicated that uptake provided three times more AdoMet than synthesis (B. Goldberg, unpublished data). Both methionine (26 nM) [8] and AdoMet (70 nM) [9] are present in human sera.

Lack of *de novo* purine synthesis, coupled with uptake of purines and purine nucleosides, is a critical feature of the metabolism of the Kinetoplastidae [10]. The characteristics of purine transporters have been examined in African trypanosomes and *Leishmania spp*. [11–14]. Carter and Fairlamb [15] have described adenosine/inosine (P_1) and adenosine/adenine (P_2) transporters in *T. b. brucei*. The P_2 transporter is also utilized by trypanocidal agents such as diamidines and melamine-based arsenical drugs [11]. In trypanosomes, the purine transporters are typical of those found in other cell types and are distinct from AdoMet transport [7]. In the earlier study, we examined several known trypanocides for uptake against both AdoMet and adenosine in *T. b. brucei*. In the present study, additional trypanocidal agents as well as intermediates and products of methionine metabolism, i.e. the polyamines putrescine, spermine, and spermidine, were examined for competition with AdoMet and with adenosine uptake in a drug-susceptible strain, *T. b. brucei* Lab 110 EATRO, and in *T. brucei rhodesiense* KETRI 243 As10-3, a diamidine- and melarsen-resistant strain cloned from a clinical isolate.

MATERIALS AND METHODS

Organisms and Culture Conditions

T. b. brucei Lab 110 EATRO, a strain susceptible to melamine-based arsenicals and diamidine trypanocides (*in vitro* and *in vivo*), and the *T. b. rhodesiense* KETRI 243 As10-3 strain (cloned from the clinical isolate KETRI 243, which is resistant to arsenical drugs and diamidines [4]), were grown as previously described [4]. In brief, both strains were grown in outbred Wistar rats (0.2-kg females, Ace Animals). Rats were killed by CO_2 anoxia, and blood was collected by cardiac puncture. Cells were separated from blood on DEAE columns, washed in 0.1 M of phosphate-buffered saline (pH 8.0) with 1.0% glucose (PBSG), and brought to a concentration of 1×10^9 cells/mL of cold PBSG. Cells were maintained in an ice bath until used. All experiments were done at room temperature (approximately 25°).

AdoMet and Adenosine Uptake

Suspensions of purified *T. b. brucei* and *T. b. rhodesiense* (10^9 cells/mL) in PBSG, were incubated with 5.5 μCi of *S*-[*methyl*-^{3}H]AdoMet (0.55 μM) or 0.2 μCi of [8-^{14}C]adenosine (26 μM). Incubation times were 0.5, 1, 2.5, 5, and 10 min. At each time point, aliquots of 0.2-mL cells (in duplicate) were centrifuged for 0.5 min through mineral oil as described by Aronow *et al.* [12], and modified by L'Hostis *et al.* [16]. Aqueous and oil layers were aspirated, and the tips of microfuge tubes were cut and analyzed by liquid scintillation spectrophotometry, in the presence of Beckman Ready Protein scintillation fluor (Beckman Instruments). Antagonism of transport by nucleosides, polyamines, nucleoside analogs, or trypanocides was carried out by including these agents in the PBSG cell suspension, starting the incubation by adding the appropriate radiolabeled substrate and sampling at the 0.5- to 10-min time points, as described above.

Effects of Specific Intermediates of AdoMet Metabolism and Analogs

The IC_{50} (growth inhibitory) concentrations of AdoMet analogs and other trypanocidal agents were tested for interference with nucleoside uptake in each strain by co-incubating with *S*-[*methyl*-^{3}H]AdoMet (0.55 μM) or [8-^{14}C]adenosine (26 μM). Most competition studies examining [^{14}C]adenosine uptake included 5 mM inosine to saturate the P_1 transporter and allow uptake through the P_2 transporter solely to be measured [11]. Specific intermediates in AdoMet utilization and methionine recycling were also examined, as well as AdoMet analogs and polyamines. Several diamidines, including pentamidine and Berenil as well as a new diamidine, CGP 40215 [17, 18], were examined for competition with AdoMet and adenosine transport, since they are AdoMet decarboxylase inhibitors that reportedly enter through the P_2 transporter [11]. The purine nucleoside analogs formycin B and dipyridamole were also examined for activity, as was the trypanocide melarsen oxide and its major organic substituent, melamine. Structures for many of these agents are given in Fig. 1.

Lysis Assays

These were carried out as previously described by Yarlett *et al.* [19]. In brief, trypomastigotes were resuspended at 2.5×10^7/mL in 1% BSA-supplemented PBSG (pH 7.4), containing (per mL) 50 units of penicillin and 50 μg of streptomycin. Suspensions were held at 4°, and aliquots were warmed to 37° prior to assay. Three-milliliter portions of trypomastigotes were added to six cuvettes containing aliquots of the arsenical, and the absorbance was monitored at 500 nm for 30 min (1-min intervals) using a thermostatically controlled recording spectrophotometer (Beckman DU7). BSA-supplemented PBSG was used as the zero absorbance reference. The time needed for 50% lysis of trypanosome isolates was determined for melarsen oxide using a range of 3–30 μM for *T. b. brucei* Lab 110 EATRO. The time point for 50% lysis with melarsen oxide at 5 μM (30 min) was used to determine the ability of nucleosides, polyamines, and other agents to protect trypanosomes from lysis. Lysis curves always included appropriate controls containing no drug, and containing melarsen oxide alone.

TABLE 1. Effect of nucleosides, polyamines, and methionine on [8-^{14}C]adenosine and *S*-[*methyl*-^{3}H]AdoMet transport in *T. b. brucei* and *T. b. rhodesiense*; protection from melarsen oxide lysis*

		T. b. brucei Lab 110 EATRO			*T. b. rhodesiense* KETRI	
		% Inhibition			% Inhibition	
	Concn (μM)	Adenosine	AdoMet	Melarsen lysis†	Adenosine	AdoMet
Adenosine	100	ND‡	0	+	ND	0
AdoMet§	100	0	ND	−	0	ND
Methionine	500	0	++‖	−	0	ND
MTA§	500	29	++‖	+	48	++‖
KMTB§	100	57	0	−	66	0
Inosine	100	16	0	−	13	0
Putrescine	1000	20	0	ND	0	0
	1000	68§	ND	ND	71§	ND
Spermidine	1000	0	0	ND	0	0
	1000	32§	ND	ND	69§	ND
Spermine	1000	0	0	ND	0	0
	1000	42§	ND	ND	22§	ND

*Transport competition studies were done as described in Materials and Methods, using rapid uptake from 0.5 to 10 min. Control rates (pmol/min/mg of protein): for adenosine, *T. b. brucei* 194.9 ± 79.4, N = 12; *T. b. rhodesiense* 69.5 ± 27.9, N = 10; for AdoMet *T. b. brucei* 1.06 ± 0.47, N = 12; *T. b. rhodesiense* 0.49 ± 0.1, N = 10. All data from adenosine, AdoMet, and inosine in *T. b. brucei* were reported previously and are included here for comparison [7].

†Protection from melarsen-induced lysis in *T. b. brucei* (+) was determined using 5 μM of melarsen oxide, as described in Materials and Methods. Compounds protecting from lysis completely blocked lysis as compared with full activity controls over a 30-min incubation.

‡ND = not determined.

§Effect on adenosine transport in the presence of inosine (5 mM).

‖Stimulated AdoMet transport.

T. b. rhodesiense KETRI 243 Clone As10-3 is completely refractory to melarsen oxide at 750 μM, and, because of this, lysis assays cannot be done with this strain.

Sources of Chemicals

S-[*methyl*-^{3}H]Adenosyl-L-methionine (64.6 Ci/mmol) and [8-^{14}C]adenosine (56 mCi/mmol) were obtained from DuPont/NEN Research Products. Iscoves modified Dulbecco's medium and all sera were from Life Technologies. HETA was synthesized as described previously [20]. CGP 40215 was a gift of Ciba–Geigy AG. MDL 73811 was a gift of Marion Merrell Dow, Inc. All other biochemicals were obtained from the Sigma Chemical Co., except for pure AdoMet, which was obtained from Research Biochemicals Int. HETA and commercial MTA were chromatographically pure (HPLC analysis) and contained no adenine or adenosine, while commercial AdoMet contained no MTA.

Calculations

All graphics were plotted on a Jandel Sigma Plot (Jandel Scientific). Experiments were performed in duplicate, and values reported are the mean inhibition of the rate of transport of multiple time points from 0.5 to 10 min. These values were determined by measuring the slope of the linear rate of uptake for control cells and cells co-incubated with the competing substance. Percent inhibition was as calculated by dividing the rate in the presence of antagonist by the control rate, and subtracting from 1.

RESULTS

Differentiation of AdoMet Transport from Adenosine Transport

To distinguish AdoMet transport from P_1 and P_2 nucleoside transport, a series of competition experiments were performed with *S*-[*methyl*-^{3}H]AdoMet (0.55 μM) or [8-^{14}C]-adenosine (26 μM). Inosine has been shown to enter through the trypanosomal P_1 (adenosine/inosine) transporter. In the presence of saturating inosine, transport through the P_2 (adenosine/adenine) transporter can be delineated from P_1 transport [11, 15]. The trypanocide melarsen oxide is taken up through the P_2 site [15]. Any agent utilizing the P_2 transport site will compete with melarsen and block melarsen-induced lysis in the susceptible *T. b. brucei* strain. The combined data from both uptake and lysis studies with nucleosides and other natural agents are presented in Table 1. Data previously reported for adenosine, AdoMet, and inosine in *T. b. brucei* are included here for comparison [7]. Inosine (100 μM) has no effect on AdoMet transport and, like AdoMet, does not protect cells from lysis in the melarsen lysis assay. Co-incubation of cells with saturating inosine (5 mM) and AdoMet (100 μM) did not further compete with adenosine transport (data not shown). These prior results in *T. b. brucei* differentiated adenosine transport through P_1 from that in P_2 as described by Carter *et al.* [11], and clearly distinguished these transporters from the system transporting AdoMet. The present results show that these essential features of P_1, P_2, and AdoMet transport are retained in *T. b. rhodesiense* KETRI 243 As10-3.

Dipyridamole is a specific inhibitor of P_2 adenosine

TABLE 2. Effect of trypanocides and nucleoside analogs on [8-^{14}C]adenosine and *S*-[*methyl*-^{3}H]AdoMet transport in *T. b. brucei* and *T. b. rhodesiense*; protection from melarsen oxide lysis*

		T. b. brucei Lab 110 EATRO			*T. b. rhodesiense* KETRI	
	Concn (μM)	% Inhibition			% Inhibition	
		Adenosine	AdoMet	Melarsen lysis†	Adenosine	AdoMet
HETA	1	30	30	+	11	42
	1	94‡	ND§	ND	74‡	ND
Pentamidine‡	100	66	14	+	81	++‖
Berenil‡	100	62	0	+	88	++‖
MGBG‡	100	80	5	+	70	++‖
MDL 73811‡	100	50	12	+	79	34
Sinefungin‡	10	19	57	−	32	61
Formycin B‡	100	71	0	−	71	0
Dipyridamole‡	100	56	69	+	87	70
CGP 40215‡	1000	56	0	+	80	0
Melarsen oxide‡	1	43	0	+	98	0
Melamine	10	48‡	0	ND	ND	ND
	1000	0	0	ND	55	0
	1000	65‡	ND	ND	83‡	ND

*Transport competition studies were done as described in Materials and Methods, using rapid uptake from 0.5 to 10 min. Control rates (pmol/min/mg of protein): adenosine *T. b. brucei* 194.9 ± 79.4, N = 12; *T. b. rhodesiense* 69.5 ± 27.9, N = 10; AdoMet *T. b. brucei* 1.06 ± 0.47, N = 12; *T. b. rhodesiense* 0.49 ± 0.1, N = 10. All data for HETA, pentamidine, Berenil, and MDL 73811 in *T. b. brucei* were reported previously and are included here for comparison [7].

†Protection from melarsen-induced lysis in *T. b. brucei* (+) was determined using 5 μM of melarsen oxide, as described in Materials and Methods. Compounds protecting from lysis completely blocked lysis as compared with full activity controls over a 30-min incubation.

‡Effect on adenosine transport in the presence of inosine (5 mM).

§ND = not determined.

‖Stimulated AdoMet transport.

transport in African trypanosomes [15] and was a potent inhibitor of adenosine uptake in both strains (Table 2). However, AdoMet uptake was also inhibited by 100 μM of dipyridamole in *T. b. brucei* (69%: 0.74 to 0.23 pmol/min/mg of protein; Table 2), and in *T. b. rhodesiense* (70%: 1.5 vs 0.47 pmol/min/mg of protein). The inhibition by dipyridamole indicates that although the AdoMet transporter is distinct from either of the adenosine transporters (P_1 or P_2), it is nevertheless a member of the nucleoside family [21].

Effects of AdoMet Analogs on AdoMet Uptake and Utilization

Sinefungin, an analog of AdoMet, is a competitive inhibitor of protein methylases [22]. When uptake of AdoMet by *T. b. brucei* was challenged by sinefungin (10 μM), the rate of transport decreased from 0.73 to 0.38 pmol/min/mg of protein (49% inhibition). At the 10-min end point, 57% inhibition of transport was obtained (Table 2). Sinefungin was not protective for *T. b. brucei* in the melarsen oxide lysis assay. AdoMet uptake by *T. b. rhodesiense* was inhibited 61% by sinefungin (1.83 vs 0.7 pmol/min/mg of protein). Sinefungin competed with adenosine for transport, inhibiting its uptake by 19% in *T. b. brucei* and by 32% in *T. b. rhodesiense*.

Formycin B, an inosine analog, enters trypanosomes through the P_1 adenosine transporter [23]. It did not protect against melarsen oxide-induced lysis in *T. b. brucei* and did not affect the rate of AdoMet transport by either strain (Table 2).

HETA is an analog of MTA and a substrate for trypanosome MTA phosphorylase [20, 24]. HETA is trypanocidal *in vitro* (IC_{50} for *T. b. brucei* is 0.54 μM; [25]) and *in vivo* cures model infections [26]. At 1 μM, HETA reduces adenosine and AdoMet uptake in *T. b. brucei* by 30% over 10 min ([7]; Table 2), reducing the rate of AdoMet uptake from 0.86 to 0.6 pmol/min/mg of protein. In the presence of saturating inosine, HETA (1 μM) inhibits adenosine uptake by 94%; HETA at 1 μM also blocks lysis of trypanosomes by melarsen oxide *in vivo* ([7]; Table 2). The *in vitro* IC_{50} value for HETA in *T. b. rhodesiense* is 0.4 μM [25]. Adenosine transport in this strain was inhibited 11% by HETA (1 μM), but in the presence of saturating inosine HETA inhibited adenosine uptake by 74% (77 vs 20 pmol/min/mg of protein; Table 2). AdoMet uptake in *T. b. rhodesiense* was inhibited 42% by 1 μM of HETA (0.92 vs 0.53 pmol/min/mg of protein). In contrast, MTA (500 μM) did not inhibit AdoMet transport in either strain (Table 1), while inhibiting adenosine transport by 29% in *T. b. brucei*, and by 48% in *T. b. rhodesiense*.

The AdoMet decarboxylase inhibitor MDL 73811 is a highly effective trypanocide *in vivo* in model infections [27]. At a low (50 μM) plasma concentration, it is rapidly concentrated and inactivates trypanosome AdoMet decarboxylase [6]. This nucleoside strongly inhibited P_2 adenosine transport in both strains (50%, *T. b. brucei*; 79% *T. b. rhodesiense*) but was less effective against AdoMet uptake (12% in *T. b. brucei*; 34% in *T. b. rhodesiense*).

Effects of Antitrypanosomal Agents on AdoMet Uptake

Pentamidine and Berenil compete for the P_2 adenosine transporter in *T. b. brucei* [11], while CGP 40215 is a

recently developed diamidine [28] that is structurally related to these conventional diamidines and which is trypanocidal *in vivo* in model infections [18]. The results in Table 2 indicate that all three diamidines compete for adenosine transport through the P_2 transporter (56–66% inhibition), in the presence of saturating inosine. Although each agent is an inhibitor of trypanosome AdoMet decarboxylase [25, 29], at 100 μM none competed with AdoMet for transport (Table 2).

MGBG, an analog of dAdoMet and an inhibitor of AdoMet decarboxylase, was strongly inhibitory to adenosine transport in *T. b. brucei* in the presence of inosine (Table 2), and was protective in the *in vitro* melarsen lysis assay. The 5% inhibition of AdoMet transport was considered insignificant. MGBG was also a potent inhibitor of P_2 adenosine transport but not AdoMet transport in *T. b. rhodesiense*. MGBG and MDL 73811 cause an increase in protein methylation and a significant increase in cytosolic accumulation of AdoMet [4, 30].

Effects of Polyamines on AdoMet and Adenosine Transport

Because the spacing of **N** and amine groups in adenosine and diamidines is similar to that of naturally occurring polyamines, adenosine uptake by *T. b. brucei* was examined in the presence of a 1-mM concentration of the polyamines putrescine, spermidine, and spermine, with and without saturating inosine (Table 1). Spermine and spermidine had no effect on adenosine transport in the absence of inosine, but in the presence of saturating inosine they caused 42 and 32% inhibition, respectively (uptake rates: spermine plus inosine, 47 vs a control rate of 82 pmol/min/mg of protein; spermidine plus inosine, 35 vs a control rate of 52 pmol/min/mg protein). Putrescine alone, however, was 20% inhibitory to adenosine transport (83 vs 67 pmol/min/mg of protein), and in the presence of inosine 68% inhibitory (83 vs 27 pmol/min/mg of protein). In *T. b. rhodesiense*, 1 mM putrescine, spermidine, and spermine had similar effects on P_2 adenosine transport: all were inhibitory in the presence of saturating inosine. Putrescine was 71% inhibitory to adenosine transport, with spermidine inhibiting 69% and spermine 22%. None of the three polyamines tested were inhibitory to AdoMet transport in *T. b. brucei* or *T. b. rhodesiense*.

Effects of Melarsen Oxide on AdoMet and Adenosine Transport

Melamine is an organic component present in the arsenicals melarsen oxide and melarsoprol (Arsobal). We therefore examined the ability of melamine to interfere with nucleoside transport. In *T. b. brucei*, which is sensitive to melarsen oxide, melamine (10 μM and 1 mM) had no effect on AdoMet transport (Table 2). Adenosine uptake was inhibited 43% by 1 μM of melarsen oxide in the presence of 5 mM of inosine (173 vs 98 pmol/min/mg of protein). Cells remained intact and motile during the short incubation period with melarsen oxide. In *T. b. brucei*, melamine in the absence of inosine was not competitive with adenosine uptake, even at 1 mM. However, melamine at 10 μM in the presence of saturating inosine caused a 48% decrease in adenosine transport (191 vs 99 pmol/min/mg of protein) and at 1 mM a 65% decrease (191 vs 67 pmol/min/mg of protein).

The *T. b. rhodesiense* KETRI 243 As10-3 clone is insensitive to melarsen oxide treatment in mouse infections and, in lysis assays, was insensitive to this agent at 750 μM, the highest concentration tested. However, adenosine uptake in the presence of saturating inosine was inhibited 98% by 1 μM of melarsen oxide (control rate of 26 vs 0.44 pmol/min/mg of protein), while melamine (1 mM) alone was 55% inhibitory (control rate of 40 vs 17 pmol/min/mg of protein). In the presence of saturating inosine, melamine (1 mM) reduced adenosine transport by 83% (40 vs 7 pmol/min/mg of protein). These results clearly indicate that melarsen oxide is transported by the resistant strain through P_2 transport.

DISCUSSION

African trypanosomes require preformed purines and acquire them through the transport of free bases and nucleosides [14]. Adenosine and inosine are the preferred substrates, while extensive interconversion of purines and their nucleosides takes place [10, 14]. Our recent studies indicated that AdoMet has a central role in trypanosome metabolism and that exogenous AdoMet can be taken up and used to directly methylate proteins and lipids [2, 4, 30]. Studies with the related kinetoplastid *Leishmania* demonstrated that exogenous AdoMet is used to methylate lipids and protein [31] and that AdoMet uptake is inhibitable by sinefungin [32]. The present investigation extends our earlier findings and confirms that transport of AdoMet is separate from adenosine and inosine transport (P_1 and P_2) in African trypanosomes.

AdoMet can be recycled to methionine and adenine/adenosine from MTA, which is formed from AdoMet during spermidine synthesis or from *S*-adenosylhomocysteine following protein and lipid methylation [33, 34]. Human serum contains approximately three times more AdoMet than methionine [8, 9], suggesting that AdoMet is a significant source of methionine through the methionine recycling pathways. AdoMet transport also appears to provide an additional mechanism for trypanosomes not only to acquire purines, but also to acquire a preformed, energetically expensive metabolite that the cell would otherwise need to synthesize.

Methodology to study nucleoside transport in mammalian tissues and parasites is most sensitive when these assays are done over a short period. This allows the use of low concentrations of radiolabeled substrate and avoids the need for saturating concentrations of substrate that would minimize the competitive effects of analogs on transport

and metabolism [35]. Following this reasoning, 0.55 μM of AdoMet and 26 μM of adenosine were used as substrate concentrations for challenge by all analogs and intermediates. At these concentrations, significant rates of transport and intracellular accumulation occur, while uptake remains highly susceptible to competition by analogs and intermediates. The percent inhibition of the rate of transport only shows the ability to compete, but does not indicate whether one analog is more competitive than another for any transporter. Only a kinetic study providing information on the effects of agents on the K_m or V_{max} values of AdoMet or adenosine transport could properly show this to be the case.

Further evidence that AdoMet and adenosine transport appear to be mutually exclusive in *T. b. brucei* was provided by examining melarsen oxide and melamine, which competed with adenosine but did not compete with AdoMet (Table 2). In a previous study, we had found that only three compounds of those tested interfered with AdoMet transport in *T. b. brucei*: dipyridamole, sinefungin, and HETA ([7]; Table 2). Related nucleosides such as MDL 73811, an analog of decarboxylated AdoMet, and MTA were not inhibitory to AdoMet transport, interfering only with adenosine uptake through P_2. In the present study, these patterns of purine nucleoside transport were extended to a highly drug-refractory *T. b. rhodesiense* clinical isolate. Although the separation of adenosine and AdoMet transport in this isolate clearly paralleled that seen in *T. b. brucei*, there were some differences in the ability of analogs to compete with uptake. While AdoMet transport in *T. b. brucei* was minimally inhibited by MDL 73811, this compound was clearly antagonistic to AdoMet uptake in the *T. b. rhodesiense* clinical isolate (34% inhibition at 100 μM; Table 2). Sinefungin inhibited adenosine uptake in *T. b. brucei* by 19%, but inhibited AdoMet by 57%. Sinefungin uptake in the clinical isolate appeared to be less specific, inhibiting adenosine transport by 32% but inhibiting AdoMet uptake by 61% (Table 2). Sinefungin is an AdoMet analog. It is slightly inhibitory of adenosine transport in the presence of inosine (Table 2), but is not protective in the lysis assay, so it does not compete effectively with melarsen oxide.

T. b. brucei Lab 110 EATRO is sensitive to melarsen oxide *in vivo* as well as *in vitro* [19]. Melarsen oxide competed with adenosine uptake in the presence of saturating inosine (1 mM, 43% inhibition) as did the arsenical carrier melamine (10 μM and 1 mM), causing up to 65% inhibition. The *T. b. rhodesiense* KETRI 243 As10-3 clone was derived from a melarsen oxide-resistant field isolate without serial passage in drug [36] and is insensitive to 750 μM in the lysis assay. However, in the presence of saturating inosine, adenosine transport in this strain was inhibited 98% by 1 μM of melarsen oxide and 83% by melamine (1 mM). Similar competition for adenosine uptake was obtained with pentamidine and Berenil. Because *T. b. rhodesiense* KETRI 243 As10-3 was shown to be resistant to pentamidine, Berenil, and melarsen oxide in model infections [18], it is surprising that these agents more avidly inhibited adenosine uptake in this isolate than in the drug-sensitive *T. b. brucei*. CGP 40215, an arylguanylhydrazone resembling a diamidine [28], also blocked adenosine uptake. However, this agent is effective as a trypanocide *in vivo* against the resistant strain [18]. These findings argue against loss or alteration of a transporter as the basis for clinically developed resistance, as previously reported for laboratory-derived resistant strains [11, 15, 18, 37, 38], and raise the possibility that there is a common feature linking melarsen oxide and pentamidine drug resistance or that multiple sites of metabolic resistance exist in clinical strains.

Putrescine, spermidine, and spermine inhibited adenosine transport in both trypanosome strains, but only in the presence of inosine. This suggests that the P_2 transporter may also function as a polyamine or amino acid transporter in African trypanosomes. Interestingly, pentamidine uptake in pathogenic *Leishmania spp*. promastigotes and amastigotes was competitively inhibited by putrescine and spermidine but not by purine or pyrimidine nucleosides [39]. Methionine salvage through MTA has been studied in cancer cells as a target for chemotherapeutically active compounds acting as either inhibitors or substrate analogs of MTA phosphorylase [33]. A similar approach in protozoan parasites has yielded one such MTA substrate analog that is trypanocidal, namely HETA, which is cleaved by the trypanosome enzyme but not as well by its mammalian counterpart. HETA is trypanocidal *in vitro*, and *in vivo* it cures model infections due to *T. b. brucei* and *T. b. rhodesiense* [24, 26]. HETA has unique uptake characteristics in drug-sensitive and drug-resistant isolates, and enters via both adenosine (P_1 and P_2) and AdoMet transporters, as opposed to MTA which is competitive only with P_2 transport. These differences in uptake of a nucleoside and its analog illustrate the potential advantage of the AdoMet site as a selective drug portal in trypanosomatids [40].

In trypanosomes, AdoMet appears to be a pivotal stress-related metabolite, whose synthesis by an unregulated AdoMet synthetase [3] and transport through its own site are meant to ensure adequate intracellular levels [7]. AdoMet pools increased 48-fold with an intracellular concentration of 1.5 mM in *T. b. brucei* treated *in vivo* for 12–36 hr with the ornithine decarboxylase inhibitor DFMO [5]. Trypanosomes incubated with [^{35}S]methionine in the presence of DFMO utilize AdoMet at a rate of 14.4 pmol/mg protein/hr as compared with 4 pmol/mg of protein/hr for control cells [4]. DFMO blocks cell division but is not cytotoxic in *T. b. brucei*. It is likely that the increase in AdoMet levels contributes to DFMO-related effects in trypanosomes and that increased AdoMet turnover in treated cells reflects increased methylation of cell components. Byers *et al.* [6] demonstrated that *T. b. brucei* treated once in rats with a single i.v. dose of the AdoMet decarboxylase inhibitor MDL 73811 develop a 20-fold increase in AdoMet within 1 hr. In a previous study [30], we demonstrated that MDL 73811 causes a 50% increase in

protein methylation, while DFMO increases lipid and protein methylation approximately two-fold. MGBG, another AdoMet decarboxylase inhibitor, also caused a two-fold increase in trypanosome protein methylation (B. Goldberg, unpublished observation). AdoHcy, the immediate product of these methylation reactions is metabolized initially to adenosine and homocysteine and, ultimately, to cystathione and cysteine, the bulk of which is excreted by bloodform trypanosomes [4]. However, the adenosine is conserved by these parasites, which do not produce adenosine *de novo*.

The presence of a unique AdoMet transporter in African trypanosomes adds another dimension to potential chemotherapy of these organisms, while the continued presence of P_2 and AdoMet transporters in a multi-drug-resistant *T. b. rhodesiense* strain indicates that mechanisms of trypanocide resistance unrelated to transport remain to be demonstrated.

AdoMet metabolism is clearly important to the normal metabolism of *Leishmania* and *Trypanosoma* species, and most likely to the numerous opportunistic parasites in HIV-positive and AIDS patients. New leads in chemotherapy targeting uptake and utilization of AdoMet may, therefore, have applicability to a wide range of protozoan parasites.

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