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IRREVERSIBLE INHIBITION OF *S*-ADENOSYLMETHIONINE DECARBOXYLASE OF *TRYPANOSOMA BRUCEI BRUCEI* BY *S*-ADENOSYLMETHIONINE ANALOGUES

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Abstract—*S*-Adenosylmethionine analogues designed as active-site directed inhibitors were tested *in vitro* for their effects on *S*-adenosylmethionine decarboxylase (AdoMetDC) of *Trypanosoma brucei brucei*. These analogues contained a tertiary nitrogen atom in place of the sulfonium and had a side chain of variable length ending in a reactive group (hydrazino-, aminooxy-, hydrazido- or a methylnitrosourea). The hydrazino- derivatives were the most potent inhibitors with IC_{50} values in the range of 40–100 nM. The most active compound (IC_{50} of 0.04 μM) was 5′-deoxy-5′-[(2-hydrazinoethyl)-methylamino]adenosine (MHZEA). Addition of MHZEA produced a time-dependent inactivation with an apparent K_i of 0.4 μM, and the enzyme half-life at a saturating concentration of MHZEA was 0.4 min. Increasing the length of the side chain or changing the methyl group attached to the nitrogen to an ethyl group reduced the potency. Replacement of the hydrazino moiety with an aminooxy group resulted in about a 30- to 35-fold decrease in inhibition potency. However, the relative order of activities of these aminooxy analogues was similar to that found in the hydrazino series with 5′-deoxy-5′-[(2-aminooxyethyl)methylamino]adenosine (MAOEA), which had an IC_{50} of 1.3 μM, being the most active. The hydrazido analogs were even less effective with 5′-deoxy-5′-[(3-hydrazino-3-oxopropyl)-methylamino]adenosine, the best inhibitor, having an IC_{50} value of 8.7 μM. The methylnitrosourea derivatives were inactive. The inactivation of trypanosomal AdoMetDC with MHZEA or MAOEA was irreversible and was greatly stimulated by putrescine, a known activator of the enzyme, indicating that the compounds bind to the active site and form a covalent bond with the enzyme. These inhibitors may have considerable potential as chemotherapeutic agents against trypanosomiasis and other protozoal infections and may also be useful in studying the role of AdoMetDC in the regulation of polyamine levels in these organisms.

Inhibition of polyamine biosynthesis has been identified as an important target for the design of therapeutic agents [1, 2]. Difluoromethylornithine, an enzyme-activated inhibitor of ornithine decarboxylase (ODC‖), has been shown to be an extremely useful clinical agent for the treatment of trypanosomiasis in humans and it also inhibits proliferation of many other protozoal parasites [2, 3]. *S*-Adenosylmethionine decarboxylase (AdoMet DC) is another key regulatory enzyme in the biosynthesis of spermidine and spermine in higher eukaryotes and spermidine in protozoa [4]. The presence of significant AdoMetDC activity has been demonstrated in many parasitic protozoa [5–7]. Berenil and Pentamidine, antitrypanosomal drugs, were found to be potent inhibitors of trypanosomal AdoMetDC [7]. Inhibition by Berenil was irreversible, while Pentamidine showed reversible inhibition of trypanosomal AdoMetDC [7]. Methylglyoxal bis-(guanylhydrazone) (MGBG) and its analogues, which are potent competitive inhibitors of AdoMetDC and cytotoxic agents, have shown curative properties against *Trypanosoma brucei* infections in mice [8], although trypanosomal AdoMetDC is less sensitive to inhibition with MGBG than the mammalian enzyme [7]. It has not yet been established to what extent the inhibition of AdoMetDC contributes to the antiparasitic actions since MGBG and Berenil as well as Pentamidine have been found to interfere with many other cell functions [9, 10]. Infection with these parasites progresses with their active proliferation within the host. In view of the role of polyamines in cell proliferation, AdoMetDC may be of greater importance in trypanosomes and, therefore, a large difference in selective inhibition may not be necessary for differential toxicity of AdoMetDC inhibitors.

More specific enzyme-activated irreversible inhibi-

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‖ Abbreviations: ODC, ornithine dedcarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; AdoMet, *S*-adenosylmethionine; Z-AbeAdo, 5′{[(*Z*)-4-amino-2-butenyl]-methylamino}-5′-deoxyadenosine; and MGBG, methylglyoxal bis(guanylhydrazone). The abbreviations used for AdoMet analogues are defined in Table 1.

Table 1. *S*-Adenosylmethionine analogues tested for the inhibition of trypanosomal *S*-adenosylmethionine decarboxylase

Class/ abbreviated name	Chemical name	R
Class I		
MHZEA	5′-Deoxy-5′-[(2-hydrazinoethyl)methylamino]adenosine	$N(CH_3)CH_2CH_2NHNH_2$
MHZPA	5′-Deoxy-5′-[(3-hydrazinopropyl)methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2NHNH_2$
MHZBA	5′-Deoxy-5′-[(4-hydrazinobutyl)methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2CH_2NHNH_2$
EHZPA	5′-Deoxy-5′-[ethyl(3-hydrazinoethyl)amino]adenosine	$N(CH_2CH_3)CH_2CH_2CH_2NHNH_2$
Class II		
MAOEA	5′-Deoxy-5′-[(2-aminooxyethyl)methylamino]adenosine	$N(CH_3)CH_2CH_2ONH_2$
MAOPA	5′-Deoxy-5′-[(3-aminooxypropyl)methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2ONH_2$
EAOPA	5′-Deoxy-5′-[(3-aminooxypropyl)ethylamino]adenosine	$N(CH_2CH_3)CH_2CH_2CH_2ONH_2$
AOPA	5′-Deoxy-5′-[(3-aminooxypropyl)amino]adenosine	$NHCH_2CH_2CH_2ONH_2$
Class III		
MHDMA	5′-Deoxy-5′-[(2-hydrazino-2-oxoethyl)methylamino]adenosine	$N(CH_3)CH_2C(O)NHNH_2$
MHDEA	5′-Deoxy-5′-[(3-hydrazino-3-oxopropyl)methylamino]adenosine	$N(CH_3)CH_2CH_2C(O)NHNH_2$
MHDPA	5′-Deoxy-5′-[(4-hydrazino-4-oxobutyl)methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2C(O)NHNH_2$
MHDBA	5′-Deoxy-5′-[(5-hydrazino-5-oxopentyl)methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2CH_2C(O)NHNH_2$
Class IV		
MMNEA	5′-Deoxy-5′-[[methyl[2-[methyl[nitroso(aminocarbonyl)]]amino]ethyl]amino]adenosine	$N(CH_3)CH_2CH_2NHC(O)N(NO)CH_3$
MMNPA	5′-Deoxy-5′-[[[3-[methyl[nitroso(aminocarbonyl)]]amino]propyl]methylamino]adenosine	$N(CH_3)CH_2CH_2CH_2NHC(O)N(NO)CH_3$

tors of AodMetDC have been synthesized [11–16]. One of these compounds, 5′{[(*Z*)-4-amino-2-butenyl]-methylamino}-5′-deoxyadenosine (*Z*-AbeAdo), cured drug-resistant *T. brucei* infections in mice and also blocked the proliferation of *Plasmodium falciparum* in cultures [14, 17]. These observations support the idea that inhibition of polyamine biosynthesis through specific AdoMetDC inhibitors may be an approach for chemotherapy of trypanosomiasis and other protozoal infections. However, with the exception of the studies with *Z*-AbeAdo, there is little information on the sensitivity of the AdoMetDC from trypanosomes to these compounds. In the present work, we have tested a series of AdoMet analogues for their ability to inhibit AdoMetDC of *T. b. brucei in vitro*. The studies may be useful in revealing structural requirements for obtaining optimum and specific inhibition of trypanosomal AdoMetDC and in understanding the mechanisms of inhibition of trypanosomal AdoMetDC by these analogues, some of which should be explored further as possible anti-trypanosomal and antiprotozoal agents.

MATERIALS AND METHODS

Materials. Putrescine dihydrochloride, *S*-adenosyl-L-methionine chloride salt and dithiothreitol were obtained from the Sigma Chemical Co. *S*-Adenosyl-L-[*carboxy*-^{14}C]methionine (47.8 mCi/mmol) was obtained from NEN. Details of the synthesis of AdoMetDC analogues listed in Table 1 have been described earlier [18, 19]. All other chemicals used were of the highest purity grade available.

Organism. *Trypanosoma brucei brucei* (isolate Lab 110/EATRO) was maintained in the laboratory by syringe passage in 200 g female Wistar rats. Trypanosomes for the preparation of enzyme extracts were isolated from heavily infected rats (72 hr post infection), purified by DEAE-cellulose chromatography [20], and washed in 50 mM Tris–HCl buffer (pH 7.4) containing 150 mM glucose and penicillin/streptomycin.

Preparation of enzyme extracts. Purified trypanosomes were suspended in 50 mM Tris–HCl (pH 7.4) containing 1 mM dithiothreitol. The trypanosomes (10^9/mL) were disrupted by freezing and thawing suspensions three times in a dry-ice methanol bath. The preparations were centrifuged for 20 min at 10,000 *g* in a refrigerated centrifuge at 4°. The supernatants containing 6–8 mg protein/mL were further subjected to ammonium sulfate fractionation. More than 90% of the AdoMetDC was recovered in the fraction that precipitated between 0 and 35% ammonium sulfate saturation. This preparation was stored at −80° in aliquots without appreciable loss of activity.

Assay of AdoMetDC. AdoMetDC was assayed by the method of Pegg and Pösö [21]. Briefly, the reaction mixture in a total volume of 250 μL contained 50 mM Tris–HCl (pH 7.4), 1.25 mM dithiothreitol, 2 mM putrescine, 0.2 mM AdoMet and 0.2 μCi of *S*-adenosyl-L-[*carboxy*-^{14}C]methionine and usually 200–400 μg of enzyme protein. For determining the specific activity of AdoMetDC, the protein content was determined by the method of Bradford [22].

Inactivation of AdoMetDC by AdoMet analogues. AdoMetDC from trypanosomes was preincubated at 37° with various concentrations of analogues in the standard assay mixture (without AdoMet) for 10 min. The reaction was started by the addition of AdoMet. For the study of time-dependent inactivation of AdoMetDC, the enzyme preparation was incubated with various concentrations of MHZEA at 37°. At various times, 20-μL aliquots were drawn and diluted into an ice-cold assay mixture. These aliquots were kept on ice until all the samples were collected. Enzyme was assayed by incubating these mixtures at 37° after addition of 0.2 μCi of labeled AdoMet. Analysis of the kinetics of AdoMetDC inhibition was performed as described by Kitz and Wilson [23].

RESULTS

The structures of the analogues of AdoMet tested for their effects on AdoMetDC of *T. b. brucei* are shown in Fig. 1 and Table 1. All compounds tested as inhibitors had a nitrogen atom in place of the sulfur in AdoMet and had a potentially reactive group at the end of an alkyl chain attached to this nitrogen. The compounds can be divided into four classes with members containing hydrazino-, aminooxy-, hydrazido- or methylnitrosourea groups. Within each class, the length of the alkyl group was varied. The abbreviations and complete chemical names for these compounds are shown in Table 1.

Compounds of classes I, II and III were inhibitory to the *T. b. brucei* AdoMetDC. The effects of

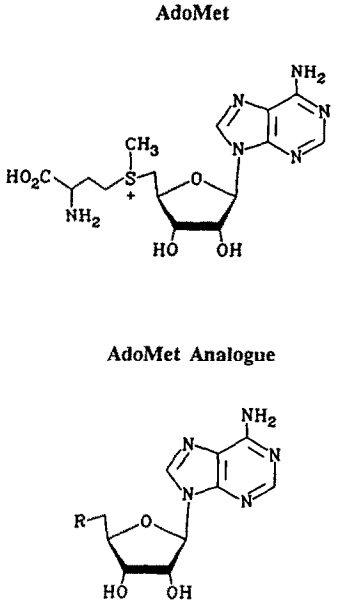

Fig. 1. Structures of AdoMet and AdoMet analogues tested for the inhibition of trypanosomal AdoMetDC. Structures of the R groups are given in Table 1.

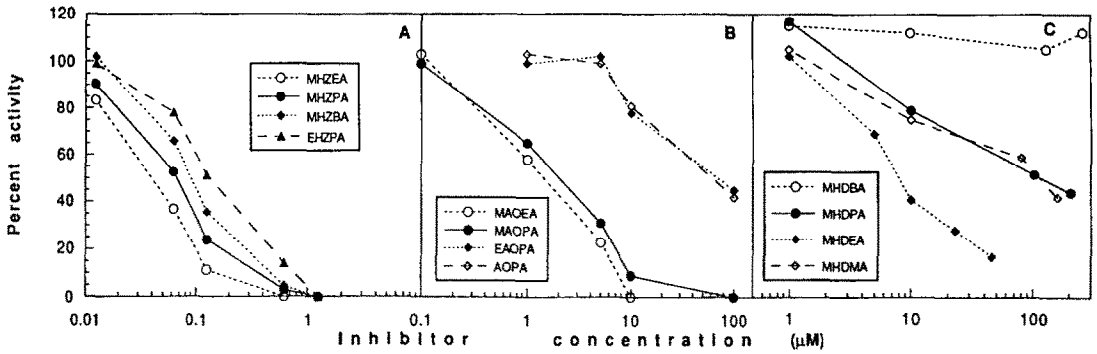

Fig. 2. Inhibition of AdoMetDC activity of *Trypanosoma brucei brucei* by some analogues of AdoMet. The trypanosomal AdoMetDC preparation was preincubated with a particular concentration of the analogue for 10 min in the standard assay mixture (without AdoMet) as described in Materials and Methods. Reaction was initiated by the addition of AdoMet and continued for 60 min. AdoMetDC activity was measured as described. Enzyme activity in the control samples was in the range of 11.5 ± 2.1 nmol/hr/mg protein.

various concentrations of these analogues on activity is shown in Fig. 2. The IC_{50} values calculated from the curves shown in Fig. 2 are given in Table 2. Analogues of class IV, MMNEA and MMNPA, were ineffective even at concentrations of up to 250 M (Table 2).

The hydrazino- compounds of class I were observed to be the most potent inhibitors of trypanosomal AdoMetDC (Fig. 2A). The strongest inhibition was obtained with MHZEA followed by MHZPA and MHZBA, showing that increasing the size of the alkyl chain decreased the activity. Substitution of the N-methyl group in MHZPA by N-ethyl (EHZPA) also caused a marked decrease in inhibition potency.

Some of the aminooxy- derivatives of class II were also effective inactivators of trypanosomal AdoMetDC. Though inhibition by these compounds was markedly lower as compared to the equivalent class I analogues, MAOEA and EAOPA were quite active. As observed with the analogues of class I, when the N-methyl group of MAOEA was substituted with N-ethyl (EAOPA), the inhibition was reduced. Removal of the methyl group to give AOPA also reduced activity (Fig. 2B).

The hydrazido- derivatives of class III were even less active as inhibitors of trypanosomal AdoMetDC. MHDEA was the best inhibitor in this series but it was >200 times less effective than MHZEA and 7 times less effective than MAOEA (Fig. 2C).

The inhibitory properties of the most potent inhibitors from classes I [MHZEA] and II [MAOEA] were studied in further detail. The inhibition by MHZEA and MAOEA could not be reversed by passing the inhibited enzyme preparation through a Sephadex G-25 column, indicating that inactivation probably involves the covalent binding of the inhibitor (Table 3). Maximal rates of inhibition of the enzyme by MHZEA and MAOEA required the presence of putrescine (Fig. 3). The presence of a high concentration (1 mM) of the substrate, AdoMet, protected the enzyme from inhibition by MHZEA (data not shown).

Table 2. Inhibition of *S*-adenosylmethionine decarboxylase of *Trypanosoma brucei brucei* by *S*-adenosylmethionine analogues

Class	Analogue	IC_{50} (μM)
I	MHZEA	0.037
	MHZPA	0.056
	MHZBA	0.088
	EHZPA	0.091
II	MAOEA	1.3
	MAOPA	1.7
	EAOPA	72
	AOPA	93
III	MHDMA	122
	MHDEA	8.7
	MHDPA	148
	MHDBA	NI
IV	MMNEA	NI
	MMNPA	NI

IC_{50} = concentration of the drug causing 50% inhibition of the enzyme. NI = no inhibition observed up to a 250 μM concentration. The assays were done in duplicates with various concentrations of the inhibitors. The IC_{50} values were computed from the inhibition curves presented in Fig. 2 after linear curve fittings. The structures of the analogues are given in Fig. 1 and Table 1.

A more detailed study of the inactivation of the trypanosomal AdoMetDC preparation by MHZEA, the most powerful inhibitor, was carried out (Fig. 4). MHZEA inactivated the trypanosomal AdoMetDC in a time- and concentration-dependent manner and the inhibition followed pseudo first order kinetics (Fig. 4A). A plot of the half-life of the activity against the reciprocal of the MHZEA concentration gave an apparent K_i value of 0.4 μM (Fig. 4B). The $T_{1/2}$ of trypanosomal AdoMetDC activity in the presence of a saturating concentration of MHZEA was 0.4 min.

Table 3. Irreversibility of inhibition of *S*-adenosylmethionine decarboxylase of *Trypanosoma brucei brucei* by MHZEA and MAOEA

	AdoMetDC activity (nmol/hr/mg protein)	
	Before gel filtration	After gel filtration
Control	11.5 ± 2.1	10.2 ± 1.9
MHZEA (0.1 μM)	2.1 ± 0.2	1.9 ± 0.2
MAOEA (5.0 μM)	3.8 ± 0.3	4.2 ± 0.4

An AdoMetDC preparation of trypanosomes was incubated with the analogues for 5 min. The preparations were passed through Pharmacia PD-10 (Sephadex G-25M) prepacked columns after taking the aliquots for AdoMetDC assay. AdoMetDC was also assayed in the elutates. Values are the means ± SD of triplicate observations.

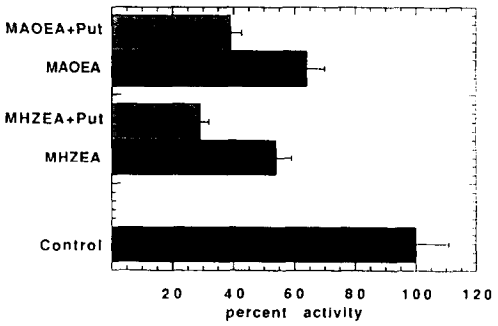

Fig. 3. Effect of putrescine on inhibition of AdoMetDC of *Trypanosoma brucei brucei* by AdoMet analogues. AdoMetDC was incubated as indicated with 0.1 μM MHZEA or 5 μM MAOEA in the presence or absence of 2 mM putrescine (Put) for 5 min and then the activity was measured. Values are means ± SD of triplicate observations. Enzyme activity in the control sample was in the range of 11.5 ± 2.1 nmol/hr/mg protein.

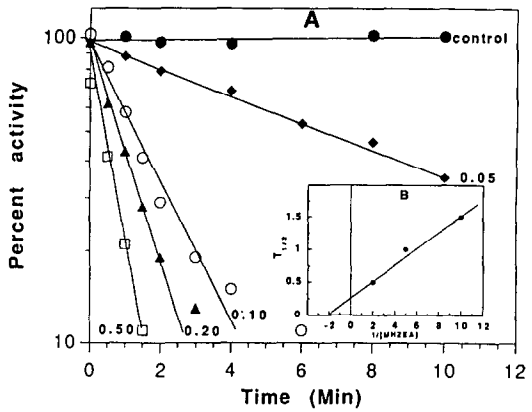

Fig. 4. Time-dependent inactivation of AdoMetDC of *Trypanosoma brucei brucei* by MHZEA. The AdoMetDC preparation of trypanosomes was incubated without or with different concentrations of MHZEA and aliquots were withdrawn at the times indicated for determination of residual AdoMetDC activity. Panel A shows the time courses for inactivation (value given with each line shows the MHZEA concentration in μM) and panel B shows the plot of $T_{1/2}$ against 1/[MHZEA] used for the determination of K_i. Enzyme activity in the control samples was in the range of 11.5 ± 2.1 nmol/hr/mg protein.

DISCUSSION

Development of specific inhibitors of enzymes of polyamine biosynthesis pathways has been an area in which major advances have been made in the past few years [reviewed in Ref. 24]. These inhibitors have provided powerful tools by which to study the physiological functions of polyamines and the regulation of their metabolism [25]. Inhibitors of ODC have been shown to be useful in the chemotherapy of cancer and protozoal infections [1–3]. It appears that AdoMetDC may also be a promising target enzyme for the design of antiprotozoal agents [6, 7, 14, 17, 26, 27]. Our results show that the trypanosomal AdoMetDC is similar to the enzyme from mammalian and bacterial sources, in that it is irreversibly inactivated with pseudo first order kinetics on incubation with AdoMet analogues containing a hydrazino- or aminooxy- group [12, 13, 16, 28]. The dependence on putrescine for a maximal rate of inactivation indicates that MHZEA and MAOPA are binding at the active site of the enzyme since putrescine is a known activator of the enzyme from mammalian, yeast and trypanosomal sources that alters the enzyme configuration to increase substrate binding [16, 29]. This inactivation indicates that the trypanosomal enzyme also contains a covalently bound pyruvate prosthetic group since binding to this group, which is present on the alpha subunit of AdoMetDC, is the basis of the inactivation of the mammalian and bacterial enzymes by the substrate and the analogues [30, 32]. This pyruvate group is generated by cleavage of AdoMetDC proenzyme at a serine residue in mammalian, bacterial and yeast AdoMetDCs [33–35]. Further evidence indicating the presence of this group in the trypanosome AdoMetDC is provided by the labeling of the enzyme by incubation with [^{35}S]-decarboxylated AdoMet in the presence of $NaCNBH_4$. This procedure forms a covalent bond by reducing the Schiff base formed between the nucleoside and the pyruvate group. Such binding was prevented in the presence of MHZEA [36]. The presence of a high concentration of the substrate, AdoMet, also prevents inhibition of the enzyme by MHZEA. Recent studies in our laboratory have confirmed the covalent binding of the closely related MHZPA at the active site of human AdoMetDC [37].

All of the compounds in classes I, II and III, which were tested as potential inactivators of AdoMetDC, contain a group (hydrazino-, aminooxy- or hydrazido-) that might be expected to form a covalent bond with the pyruvate prosthetic group if placed in close proximity to it at the enzyme active site. Previous work has shown that the deoxyadenosine moiety of AdoMet is needed for such binding [11, 31] and that binding is maintained if the sulfonium center can be replaced by a nitrogen [11, 31]. Therefore, all of the compounds tested in the present studies contained a side chain attached to a nitrogen atom linked to the 5′-position of deoxyadenosine. The results indicate that the

presence of a hydrazino- group gives the most potent inhibitors followed by the aminooxy- group, and that the compounds containing a hydrazido- group are the least effective. This observation may be explained by the relative order of reactivity of these compounds with the pyruvate carbonyl group. A similar order of sensitivity applies to the rat prostate AdoMetDC, which is almost an order of magnitude more sensitive to MHZPA as compared to MAOEA [12].

The methylnitrosourea derivatives of class IV were not effective inhibitors. These compounds would be expected to generate a reactive species at the active site and the absence of inhibition may indicate a lack of suitable essential residues for reaction with this species. However, it is also possible that the size of these compounds prevents adequate binding to the active site. The length of the alkyl chain is clearly an important factor in determining the potency of compounds of classes I and II against the trypanosome AdoMetDC. The shorter chain compounds, MHZEA and MAOEA, were the most active and activity decreased as the length of the chain increased. This may indicate a steric restraint at the active site.

The presence of a methyl group attached to the nitrogen replacing the sulfur was an important factor in the binding of the inhibitors to trypanosome AdoMetDC. Replacing this by an ethyl group reduced the effectiveness (compare EHZPA with MHZPA and EAOPA with MAOPA). Also AOPA, in which the methyl group was removed, was much less active than MAOPA. However, the methyl group was not as important in the inactivation of the mammalian enzyme by these inhibitors since MHZPA and EHZPA had similar potencies as did MAOPA and AOPA [12].

In general, the trypanosome AdoMetDC was less sensitive to inhibition by compounds in classes I and II than the human enzyme (Tekwani BL, Secrist JA III, and Pegg AE, unpublished observations). One exception was MHZEA, which is a more potent inhibitor of trypanosomal AdoMetDC. MHZEA exhibited an IC_{50} value of 87 nM for human AdoMetDC, which was more than 2-fold higher than shown for trypanosomal enzyme (37 nM).

Although our results indicate that the most active compounds for *in vitro* inhibition of AdoMetDC activity are the hydrazino- derivatives and that a methyl group attached to the nitrogen is preferred, it remains to be seen whether these compounds have optimal *in vivo* stability and specificity. It may be necessary to sacrifice some potency in order to obtain compounds with the most desirable properties for pharmacological use. However, preliminary data suggesting *in vivo* efficacy and stability of some of these AdoMet analogues (viz. MHZPA and MAOEA) as well as related AdoMet analogues have already been established by their effects on the lowering of polyamine levels and their cytotoxic effects in mammalian cell cultures [12, 13, 28].

MHZEA is the most potent inhibitor of trypanosomal AdoMetDC reported so far with a K_i value of 0.4 μM, which is significantly lower than that of 1.5 μM reported for the Z-AbeAdo (MDL73811) [14]. The structure–activity analysis of inhibition of the trypanosomal enzyme by AdoMet analogues shows that, although the protozoal enzyme shares many common characteristics with the mammalian counterpart, it also differs significantly in terms of relative sensitivity towards inhibition by these analogues. MHZEA, which showed better inhibition of trypanosomal enzyme than the mammalian AdoMetDC, is clearly worthy of further testing to determine whether it has an effect on the proliferation of trypanosomes *in vitro* as well as the cure of model infections. However, our results also indicate the possibility that differences in the active site between the mammalian and trypanosomal AdoMetDCs may allow the design of even more selective inhibitors.

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