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IRREVERSIBLE INHIBITION OF RAT *S*-ADENOSYLMETHIONINE DECARBOXYLASE BY 5′-{[(*Z*)-4-AMINO-2-BUTENYL]METHYLAMINO}-5′-DEOXYADENOSINE

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Abstract—5′-{[(*Z*)-4-Amino-2-butenyl]methylamino}-5′-deoxyadenosine ((*Z*)-AbeAdo) was tested *in vitro* and *in vivo* as a potential inhibitor of *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), a pyruvoyl-containing enzyme, purified from rat liver. *In vitro* (*Z*)-AbeAdo produces a time- and dose-dependent irreversible inhibition of the enzyme. Saturation kinetics are observed when the enzyme is preincubated with (*Z*)-AbeAdo in the presence of 50 μM putrescine, a known activator of AdoMetDC. Under these conditions kinetic constants were measured ($K_i = 0.56 \pm 0.04$ μM; $\tau_i = 0.51 \pm 0.03$ min). The inhibition is not relieved by prolonged dialysis of the inactivated enzyme. The turnover number for (*Z*)-AbeAdo, i.e. the number of inactivator molecules required to inactivate one enzyme molecule, is approximatcly 1.5. The selectivity of (*Z*)-AbeAdo was explored: the compound is not a substrate of adenosine deaminase, mitochondrial monoamine oxidase and diamine oxidase, but is slowly oxidized by benzylamine oxidase from rat aorta. The (*E*)-isomer of AbeAdo, is at least 100-fold less active than (*Z*)-AbeAdo as a time-dependent inhibitor of rat liver AdoMetDC. In rats, intraperitoneal administration of (*Z*)-AbeAdo produces a rapid, long-lasting and dose-dependent decrease of AdoMetDC activity in ventral prostate, testis and brain.

Considerable experimental evidence accumulated during the last two decades led to the concept that the polyamines putrescine, spermidine and spermine play essential functions in cellular growth and differentiation [1, 2]. Much of this progress has been due to the use of specific inhibitors to interfere with polyamine metabolism, and more particularly with inhibitors of ornithine decarboxylase (EC 4.1.1.17), the enzyme responsible for the biosynthesis of putrescine. This was well illustrated by the striking efficacy of the ornithine decarboxylase inhibitor α-difluoromethylornithine in retarding the growth of malignant cells and in treating important protozoan diseases [1]. While ornithine decarboxylase inhibitors produce a decrease of putrescine and, to a lesser extent, of spermidine concentrations in cells in culture and in animal tissues, spermine concentrations are barely affected [2–4]. The actual rate-limiting step in the biosynthesis of spermidine and spermine is catalysed by *S*-adenosyl-L-methionine decarboxylase (AdoMetDC,† EC 4.1.1.50), a pyruvoyl-containing enzyme [5, 6]. A number of reversible and irreversible AdoMetDC inhibitors have been reported [2]. However, they were neither sufficiently potent, nor sufficiently stable, nor sufficiently selective to permit investigation of AdoMetDC importance in cellular physiology *in vivo* [2]. An enzyme-activated irreversible inhibitor may fulfil these requirements.

Recently, we designed 5′-{[(*Z*)-4-amino-2-butenyl]methyl-amino}-5′-deoxyadenosine ((*Z*)-AbeAdo) as a potential enzyme-activated irreversible inhibitor of AdoMetDC and we showed that it is a potent inhibitor of the *Escherichia coli* enzyme [7]. Here, we show that (*Z*)-AbeAdo is also an extremely potent and effective inhibitor of mammalian AdoMetDC, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals. *S*-Adenosyl-L-[carboxyl-^{14}C]methionine (59 Ci/mol) was purchased from the Radiochemical Centre (Amersham, U.K.); *S*-adenosyl-L-methionine, methylglyoxal-bis(guanylhydrazone) (MGBG), and epoxy activated Sepharose 6B were purchased from Sigma (St Louis, MO, U.S.A.). (*Z*)-AbeAdo, (*E*)-AbeAdo and 5′-(dimethylsulfonio)-5′-deoxyadenosine were synthesized as reported previously [7, 8]. All other chemical products were of the purest grade commercially available.

Synthesis of MGBG-Sepharose. MGBG-Sepharose, used for affinity chromatography in the AdoMetDC purification, was obtained by incubating epoxy activated Sepharose 6B with a 0.1 M MGBG solution (pH 9.0) for 24 hr at 37° [9].

Animals. Male Sprague–Dawley rats (200–300 g body wt) were purchased from Charles River (Saint-Aubin-les-Elbeuf, France). Animals had access to a standard diet *ad libitum* and were kept on a constant 12 hr light/12 hr dark schedule. Treated animals were injected intraperitoneally with the inhibitor dissolved in 0.9% NaCl and killed by decapitation.

Preparation of tissue extracts. After the death of

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† Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; MAO, monoamine oxidase; MGBG, methylglyoxal-bis-(guanylhydrazone); (*Z*)-AbeAdo; 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine; (*E*)-AbeAdo, 5′-{[(*E*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine.

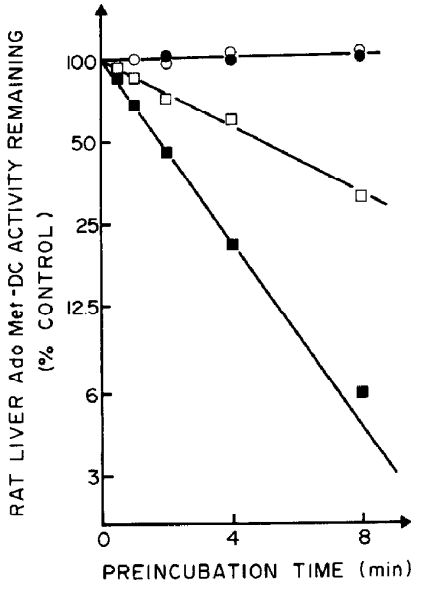

Fig. 1. Time-dependent inhibition of rat liver AdoMetDC by (Z)-AbeAdo. AdoMetDC was incubated at 37° in Tris–HCl buffer (10 mM, pH 7.5) containing 2.5 mM dithiothreitol, 0.1 mM EDTA and the inhibitor, in the presence or in the absence of putrescine (○: no inhibitor, no putrescine; ●: no inhibitor, 50 μM putrescine; □: 0.2 μM (Z)-AbeAdo, no putrescine; ■: 0.2 μM (Z)-AbeAdo, 50 μM putrescine).

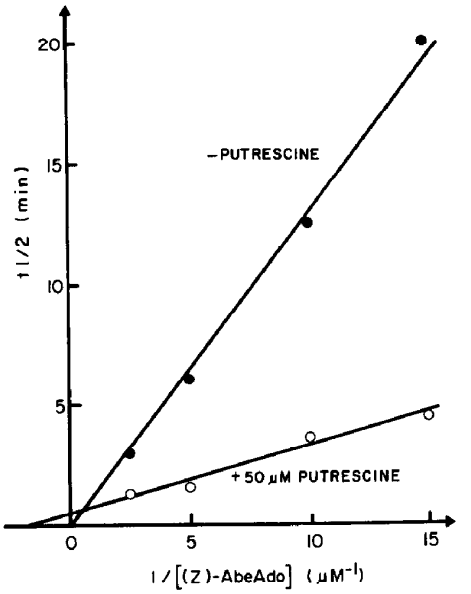

Fig. 2. Kitz and Wilson replots of the time-dependent inhibition of rat liver AdoMetDC by (Z)-AbeAdo, in the absence and in the presence of 50 μM putrescine. Times of half-inactivation (t_i) are plotted against the reciprocal of the inhibitor concentration.

the animals, tissues were homogenized with three vol. of 30 mM sodium phosphate buffer, pH 7.1, containing 5 mM dithiothreitol, 0.25 M sucrose and 0.1 mM EDTA. AdoMetDC activities were determined as described by Pegg and Williams-Ashman [10] on tissue homogenates.

AdoMetDC purification. Rat liver AdoMetDC was purified essentially as described by Pegg [11] starting from the livers of rats which had been injected with a single dose of MGBG (80 mg/kg of body weight) 22 hr before death. After a single MGBG-Sepharose step, the last step was performed by FPLC using a 5 mm × 5 cm Mono-Q anion exchange column (Pharmacia, Uppsala, Sweden). The enzyme was eluted with a NaCl gradient (0–0.3 M) in 30 min. The specific activity of the preparation was 710 nmol of CO_2/min/mg of protein when measured at pH 7.1 under the assay conditions of Pegg and Williams-Ashman [10].

Protein determination. During the purification, protein was determined by the method of Bradford [12] using Bio-rad reagents. Bovine serum albumin (BSA) was used as standard.

Assay of time-dependent inhibition of AdoMetDC. For a typical experiment, 315 μL of enzyme preparation in solution in Tris, HCl buffer (10 mM, pH 7.5), containing 2.5 mM dithiothreitol and 0.1 mM EDTA was mixed at time zero with 35 μL of a solution of inhibitor in water, and was incubated at 37° in a shaking water bath. At various times, 50-μL aliquots were transferred into a 950-μL assay medium containing sodium phosphate buffer (0.1 M, pH 7.1), 1 mM putrescine, 5 mM dithiothreitol, 0.2 mM AdoMet and 0.1 μCi radiolabelled AdoMet. The reaction was allowed to proceed for 30 min at 37°.

Other enzymes. Adenosine deaminase (EC 3.5.4.4.) from calf intestinal mucosa (Type VIII) purchased from Sigma, was assayed according to the method of Murphy *et al.* [13]. Partially purified mitochondrial monoamine oxidase (MAO, EC 1.4.3.4) from rat brain and rat small intestine diamine oxidase (EC 1.4.3.6) were prepared according to Snyder and Hendley [14]. These two enzymes, as well as hog kidney diamine oxidase (Sigma) were assayed according to Ref. 14. Rat aorta benzylamine oxidase (EC 1.4.3.-, also referred to as semicarbazide-sensitive amine oxidase [15]) was prepared as described by Lyles and Fitzpatrick [15] and was also assayed according to Ref. 14. Rat liver ornithine decarboxylase was prepared and assayed as described previously [16].

RESULTS AND DISCUSSION

Inactivation of rat liver AdoMetDC by (Z)- *and* (E)-*AbeAdo*

Incubation of purified rat liver AdoMetDC with (Z)-AbeAdo in the absence or in the presence of putrescine, a powerful activator of mammalian AdoMetDC *in vitro* [10, 17], resulted in a time-dependent loss of enzyme activity which followed pseudo first-order kinetics (Fig. 1). Loss of activity was related to the concentration of inhibitor. By plotting the time of half-inactivation (t_i) as a function of the reciprocal of the inhibitor concentration ($1/I$) according to the method of Kitz and Wilson [18], a straight line was obtained. In the absence of putrescine in the preincubation medium, this line passed through the origin (Fig. 2). However, in the presence of 50 μM putrescine, the line did not pass through the origin but intercepted the positive y-axis, demonstrating a saturation effect which involves the enzyme active-site in the inhibitory process (Fig. 2).

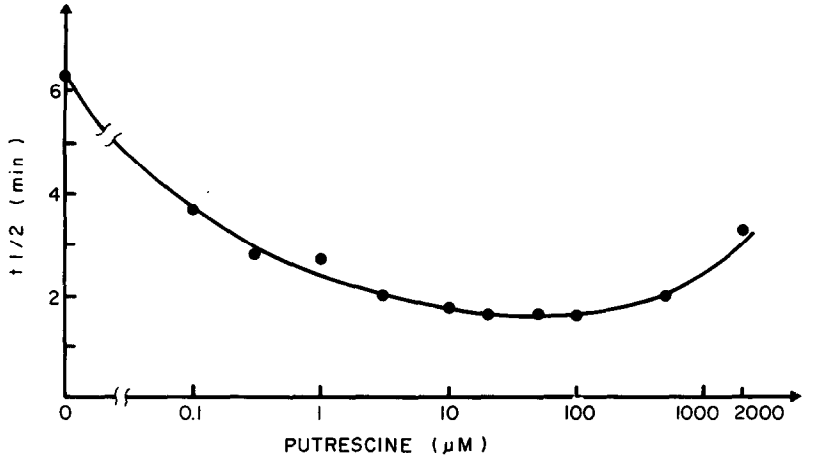

Fig. 3. Effect of putrescine on the inactivation of rat liver AdoMetDC by (*Z*)-AbeAdo. AdoMetDC was incubated at 37° in Tris–HCl buffer (10 mM, pH 7.5) containing 2.5 mM dithiothreitol, 0.1 mM EDTA and 0.2 μM (*Z*)-AbeAdo in the presence of various concentrations of putrescine. Times of half-inactivation ($t_{\frac{1}{2}}$) are plotted as a function of the putrescine concentration.

Table 1. Inactivation of rat liver AdoMetDC by (*Z*)-AbeAdo:protection by competitive inhibitors

Addition to preincubation medium	Time for half-inactivation (min)
None	>200
0.1 μM (*Z*)-AbeAdo	15
0.1 μM (*Z*)-AbeAdo + 20 μM MGBG	>200
0.1 μM (*Z*)-AbeAdo + 200 μM 5′-(dimethylsulfonio)-5′-deoxyadenosine	100

Under these conditions, kinetic constants for the time-dependent inhibition of rat liver AdoMetDC, i.e. the apparent dissociation constant (K_I) and the time of half-inactivation to infinite concentration of inhibitor ($\tau_{\frac{1}{2}}$), were extrapolated from such a Kitz and Wilson replot ($K_I = 0.56 \pm 0.04$ μM; $\tau_{\frac{1}{2}} = 0.51 \pm 0.03$ min). Effects of putrescine on the inactivation of rat liver AdoMetDC by (*Z*)-AbeAdo were examined in more details. The time of half-inactivation of the enzyme by (*Z*)-AbeAdo was decreased as a function of putrescine concentration with a maximal effect occurring between 10 and 100 μM of the diamine. At these concentrations, the $t_{\frac{1}{2}}$ value was found to be four times lower than in the absence of putrescine (Fig. 3). At higher concentrations, the effect was less pronounced and, at 2 mM putrescine, the $t_{\frac{1}{2}}$ value was only half of the control. This result shows that, as expected, putrescine stimulates the inactivation process even if it can compete with (*Z*)-AbeAdo at very high concentrations.

Further studies on rat liver AdoMetDC inactivation by (*Z*)-AbeAdo showed protective effects of the competitive inhibitors MGBG [19] and 5′-(dimethylsulfonio)-5′-deoxyadenosine [8], as shown in Table 1. This confirms that the inactivation produced by (*Z*)-AbeAdo takes place in the enzyme active-site. Furthermore, the presence of 2.5 mM dithiothreitol in the preincubation medium, as well as the absence of a lag-time before the onset of inhibition, rules out the possibility that the species responsible for inactivation was released from the enzyme active-site [20]. Incubation with 0.5 μM (*Z*)-AbeAdo for 22 min at 37° resulted in 99% inactivation of AdoMetDC. Prolonged dialysis of the inactivated enzyme for 24 hr at 4° did not produce any recovery of enzyme activity, suggesting the formation of a covalent linkage of the inhibitor to the active-site of AdoMetDC.

The (*E*)-isomer of AbeAdo was found to be substantially less potent than the (*Z*)-isomer to produce inactivation of AdoMetDC. For instance, the time of half-inactivation of the enzyme was about 80–100 min when AdoMetDC was preincubated with 10 μM (*E*)-AbeAdo (data not shown).

Determination of the partition ratio

Theoretical specific activity of the pure rat liver AdoMetDC was previously estimated to be 3000 units/mg of protein by Shirahata *et al.* [21]. Based on this value and on the activity of our preparation, we could calculate the concentration of active AdoMetDC, assuming that the enzyme has two subunits of M_r 32000 and two subunits of M_r 6000 [22]. Incubation of 150 nM AdoMetDC for 60 min with various concentrations of (*Z*)-AbeAdo ranging from zero to 200 nM produced time-dependent inactivation of the enzyme which reached plateaux when the inhibitor was entirely consumed (not shown). A plot of the percentage of enzyme activity remaining vs the ratio

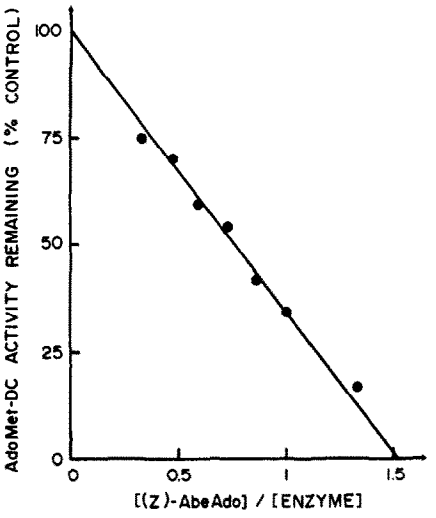

Fig. 4. Determination of the turnover number for the inactivation of AdoMetDC from rat liver by (Z)-AbeAdo. AdoMetDC (150 nM) was incubated for 60 min at 37° with various concentrations of (Z)-AbeAdo ranging from zero to 200 nM. Percentage of enzyme activity remaining was plotted vs the ratio of the moles of inactivator per mole of enzyme subunit.

of the moles of inactivator per mole' of enzyme subunit was constructed (Fig. 4) according to Silverman [23]. Thus, the turnover number (i.e. the number of inactivator molecules required to inactivate one enzyme molecule) could be extrapolated and was found to be approximately 1.5. Consequently, the partition ratio is close to 0.5 [23].

Selectivity of (Z)-*AbeAdo* in vitro

When assayed at 1 mM, (Z)-AbeAdo was not found to be a substrate of adenosine deaminase in spite of the fact that the compound is an adenosine derivative. Furthermore, at the same concentration, (Z)-AbeAdo is neither a substrate of rat brain MAO or of diamine oxidases from hog kidney and from rat small intestine, nor an inhibitor of rat liver ornithine decarboxylase in spite of the fact that it is a putrescine derivative in some respects. Nevertheless, (Z)-AbeAdo was found to be slowly oxidized by benzylamine oxidase prepared from rat aorta: when (Z)-AbeAdo and benzylamine were tested at 5 μM (a concentration equal to the K_M value of benzylamine for the enzyme), the rate of oxidation of (Z)-AbeAdo was found to be 15% of that of benzylamine.

Effect of (Z)-*AbeAdo on rat tissues* ex vivo

As shown in Fig. 5, maximum inhibition of AdoMetDC in the ventral prostate occurred 6 hr after a single intraperitoneal injection of 100 mg/kg of body weight (Z)-AbeAdo. At this time, the remaining AdoMetDC activity was 6–7% of the control value in this tissue. Thereafter, enzyme activity increased to reach about 60% of the control value 12 hr after the injection and remained at this level 24 hr after the injection. The time-course of AdoMetDC activity in testis and brain was similar to that observed in prostate, but maximum inhibition was less pronounced than in this tissue. Figure 6 shows that the decrease of AdoMetDC activity produced by (Z)-AbeAdo was dose-dependent in the ventral prostate

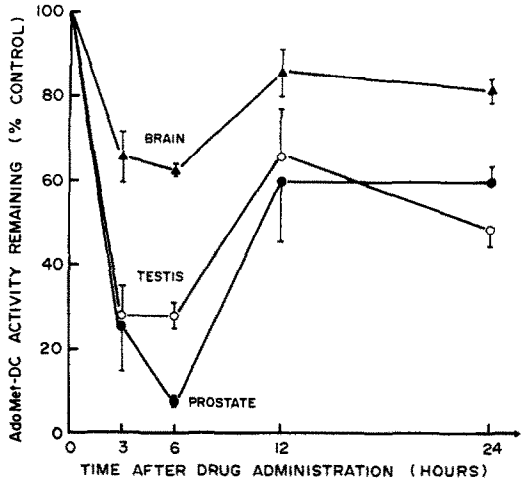

Fig. 5. Effect of a single injection of 100 mg/kg body wt (Z)-AbeAdo on AdoMetDC in ventral prostate, testis and brain. (Z)-AbeAdo was administered i.p. to rats at time zero. At given intervals, animals were killed and enzyme activities were immediately measured. Each value is the mean ± SE of five animals.

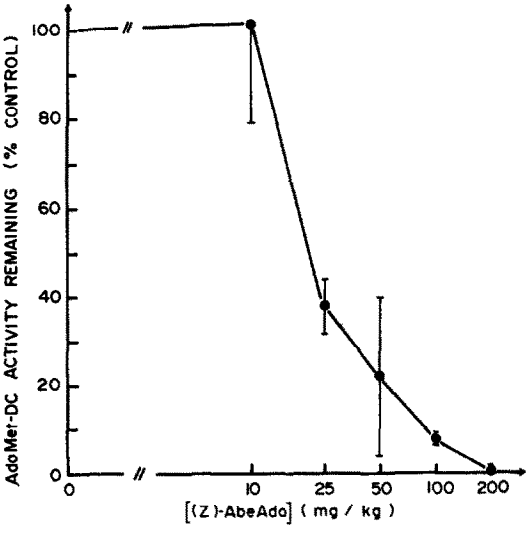

Fig. 6. Effect of a single injection of (Z)-AbeAdo on AdoMetDC activity in ventral prostate. AdoMetDC activity was measured 6 hr after i.p. administration of (Z)-AbeAdo to rats. Each value is the mean ± SE of five animals.

and that 6 hr after a single intraperitoneal injection of 200 mg/kg of body weight (Z)-AbeAdo, inhibition of AdoMetDC was almost total.

CONCLUSION

It appears from this study that (Z)-AbeAdo is a potent and selective time-dependent inhibitor of rat AdoMetDC *in vitro* and our data are consistent with this compound being an effective enzyme-activated irreversible inhibitor (or mechanism-based inactivator [23]). We had shown previously that (Z)-AbeAdo was also a potent enzyme-activated irreversible inhibitor of the *Escherichia coli* enzyme [7].

It is quite remarkable that the same compound inhibits two enzymes which, while containing pyruvate as a prosthetic group, differ in many respects [5]. More particularly, it has been demonstrated recently that there is little if any similarity between the sequence of the mammalian enzyme and that of the bacterial AdoMetDC [24].

Finally, the capability of (*Z*)-AbeAdo to inhibit AdoMetDC in tissues as different as ventral prostate, testis and brain makes the compound a promising tool to evaluate AdoMetDC importance in cellular physiology *in vivo*. The interesting properties of (*Z*)-AbeAdo warrant an examination of its pharmacological and therapeutical properties [25].

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