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ADENOSINE ANALOGUES AS INHIBITORS OR INACTIVATORS OF S-ADENOSYLHOMOCYSTEINE HYDROLASE FROM BOVINE PANCREAS

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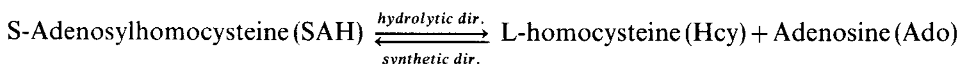
The ability of some substrate-analogues to inhibit or to inactivate S-adenosylhomocysteine hydrolase (SAHase) purified from bovine pancreas was investigated. Our results confirm that 3-deazaarysteromicin (DZAry) is a more potent competitive inhibitor than 3-deazaadenosine (DZA), while nebularine (purine riboside), contrary to previous reports, showed an uncompetitive inhibition. Moreover, 2-chloroadenosine and 2′-deoxyadenosine were found to be irreversible inactivators of SAHase with increasing potency, respectively. K_i values found for these drugs were of the same order of magnitude as those reported for SAHases from other mammalian tissues. The SAHase substrate-analogues studied are believed to act as antineoplastic and/or antiviral agents. It is conceivable to postulate that their therapeutic effects could be, at least in part, attributable to inhibition or even to inactivation of SAHase which, in turn, causes a reduction in S-adenosylmethionine-dependent methylation reactions.

KEY WORDS: Adenosine analogues, inhibitors and inactivators of S-adenosylhomocysteine hydrolase, bovine pancreas enzyme, antiviral compounds.

INTRODUCTION

S-adenosylhomocysteine (SAH) is the product of all the biological methylations in which S-adenosylmethionine is utilized as the methyl donor. This relevant metabolite is reversibly hydrolysed to L-homocysteine (Hcy) and adenosine (Ado)[1] by S-adenosylhomocysteine hydrolase (SAHase, EC 3.3.1.1) according to the following scheme:



$$\text{S-Adenosylhomocysteine (SAH)} \underset{\textit{synthetic dir.}}{\overset{\textit{hydrolytic dir.}}{\rightleftarrows}} \text{L-homocysteine (Hcy)} + \text{Adenosine (Ado)}$$

Although the equilibrium this reaction is far in the direction of synthesis, *in vivo*, the process is forced to proceed towards the hydrolysis (i.e. formation of Ado and

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Hcy) because both products are efficiently removed and converted in further metabolic pathways.[2-4]

Accumulation of SAH both *in vivo* and *in vitro*, leads to a feedback inhibition of transmethylating reactions.[2,5-8] Thus all methyltransferases utilizing adenosylmethione as the methyl donor are competitively inhibited by its product (SAH) and, in most cases, quite drastically.[9] It has also been postulated that SAH might be involved in the severe combined immuno-deficiency disease found in patients with adenosine deaminase deficiency.[10,11]

SAHase has been purified to homogeneity or partially purified from different sources.[12-14] The native enzyme from mammalian tissues has a molecular weight ranging between 180–230 kDa and, as first reported by Richards *et al.*,[15] exists as a tetramer containing 4 moles of NAD^+ bound per mole of hydrolase. The mechanism of the catalytic cycle, based on oxidation-reduction of the enzyme-bound NAD^+ has been elucidated.[16]

Several nucleoside analogues interact with SAHase serving either as substrates or as inhibitors and/or inactivators with various degrees of potency.[17,18]

In this paper we report a comparative study of the ability of some of these substrate-analogues to act as inhibitors or inactivators of SAHase purified to homogeneity from bovine pancreas. The selection of such compounds was made in view of their possible therapeutical use mainly as antineoplastic or antiviral agents.

MATERIALS AND METHODS

Chemicals

N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), Tris(hydroxymethyl)-aminomethane (Tris-HCl), D,L-homocysteine, adenosine, inosine, 8-bromoadenosine, purine ribonucleoside (nebularine), 7-deazaadenosine (tubercidin), 2′-deoxyadenosine (2′-dAdo), 2-chloroadenosine (2-Cl-Ado) were from Sigma Chemical Co. (St. Louis, MO). Adenosine deaminase (ADA EC 3.5.4.4.) from calf intestine (332 U/mg proteins), S-adenosyl-L-homocysteine, 1,4-dithio-DL-threitol, 2-mercaptoethanol were from Boehringer Biochemia, (Mannheim, Germany). 1-3,Diaminopropane, glycerol, ammonium sulfate, perchloric acid, potassium chloride and other chemicals were from Merck (Darmstadt, Germany). Acrylamide, N′N′-methylene-bisacrylamide, ammonium persulfate, tetramethylenediamine (TEMED) and bromophenol blue were from Bio-Rad (Richmond, CA). Sephadex G-50, G-10 and CH-Sepharose-4B were from Pharmacia Fine Chemicals, (Uppsala, Sweden). Protein concentrations were estimated by the Pierce protein assay reagent (product no. 23200). 3-deazaadenosine (DZA) and 3-deazaarysteromicin (DZAry) were kindly supplied by Dr P.K. Chiang, Walter Reed Army Institute of Research, (Washington, U.S.A.).

Enzyme Purification

SAHase was purified from bovine pancreas according to Kajander.[12] The degree of purity was assessed by SDS/PAGE. 1U of enzyme catalyzed, at 37 °C, in the hydrolytic direction, the cleavage of 1 nmol SAH/mg per min; while, in the synthetic direction, 1U SAHase was equivalent to 1 nmol SAH produced/mg per min.

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SAHase Activity in the Hydrolytic Direction (Without or With Inhibitors)

SAHase activity in the hydrolytic direction was monitored spectrophotometrically by a coupled assay method.[19] 3 μg of purified SAHase were incubated 5 min at 37 °C in 0.1 M Hepes, pH 7.2, 1 mM EDTA, 1 mM DTT, increasing amounts of SAH (1 ml final volume) in the absence or in the presence of the selected inhibitor. The reaction was started by addition of an excess of adenosine deaminase (2U), monitoring spectrophotometrically the absorbance decrease at 265 nm for 4–5 min. An ε_{mM} of 7.76, based on the differences at 265 nm of ε_{mM} between adenosine and inosine, was utilized for calculating the enzyme activity (nmoles/mg per min).

Time Dependent Inactivation Studies (Hydrolytic Direction)

3 μg of SAHase were incubated at 37 °C in 0.1 M Hepes, pH 7.2, 1 mM EDTA, 1 mM DTT in the absence or in the presence of the selected inactivator. The reaction was started by adding 50 μM SAH and 2U ADA to a final volume of 1 ml. To demonstrate the irreversible inactivation of SAHase, the incubated mixture was extensively dialysed against 30 mM potassium phosphate, pH 7.3 and the activity assayed again.

HPLC Monitoring of SAHase Activity in the Synthetic Direction (in the Absence or in the Presence of Inhibitors)

SAHase activity in the synthetic direction was followed by HPLC, according to de la Haba *et al.*[20] with minor modifications. 1 μg of SAHase was incubated at 37 °C, in 30 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA and 2 mM D,L-Hcy with or without the selected inhibitor. The reaction was initiated by adding 2-100 μM adenosine (final volume of 0.5 ml); after 10 min incubation, the reaction was stopped by addition of 40 μl ice-cold 3N PCA. Upon standing 2 min in ice, the mixture was neutralised by adding 30 μl of 3M potassium carbonate solution. The precipitated material was discarded by 5 min centrifugation at 20,000 × g, at 4 °C. 25–50 μl of the cleared supernatant were injected into an LC-18T Supelco cartridge connected to a Beckmann HPLC apparatus. The elution of SAH, monitored at 260 nm, was obtained by an isocratic buffer consisting of 50 mM potassium phosphate, pH 4.3, 25 mM NaCl and 2.75% methanol at a flow rate of 0.8 ml/min. The retention time of SAH was 3 min. The quantitative evaluation of peaks of SAH was performed by comparing the heights of each peak with that of a suitable standard curve obtained by injecting known amounts of authentic SAH. 1U of enzymatic activity was equivalent to 1 nanomole of SAH synthesised/mg.per min. In time-dependent inactivation studies (synthetic direction) 1 μg of SAHase was incubated at 37 °C in 30 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA with or without the selected inactivator. At the reported times, the reaction was initiated by adding 50 μM Ado and 2 mM D,L-Hcy (in 0.5 ml final volume). After 10 min, the reaction was stopped and treated as described in the above paragraph. Values of K_i were obtained using the graphical method proposed by Ascenzi *et al.*;[21] an average value ($\pm$8%) was evaluated for K_i values.

RESULTS

The kinetic parameters obtained by studying the SAHase-catalyzed reaction either in the hydrolytic or in the synthetic direction are shown in Table 1. These values are in

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Table 1 Kinetic parameters of SAHase for the reaction catalyzed in the hydrolytic and in the synthetic direction

SAHase activity	K_m (μM)	V_{max} (nmoles/min/mg)
Hydrolytic direction (SAH)[a]	42.0	4.50
Synthetic direction (Ado)[b]	8.0	0.98

[a]Spectrophotometric assay. [b]HPLC assay.

Table 2 K_i values of the different inhibitors assayed on SAHase in the hydrolytic direction

SAHase inhibitors	K_i (M)
DZAry	1.0×10^{-8}
DZA	1.0×10^{-6}
Nebularine	1.8×10^{-5}

good agreement with similar data reported by other authors[22,23] for enzymes purified from sources different from bovine pancreas. When SAHase was assayed in the hydrolytic direction in the presence of various inhibitors, their influence on the activity of adenosine deaminase (a necessary ingredient in the coupled assay reaction) was checked. Under our experimental conditions, none of the adenosine analogues tested displayed a significant inhibition againt ADA. As shown in Figure 1 (panels A and B) the kinetic profile for two inhibitors investigated (DZA and DZAry) confirms the competitive-type of inhibition, and a more powerful effect exerted by DZAry on bovine pancreas SAHase with respect to other mammalian hydrolases. On the other hand, nebularine, a purine riboside, previously reported as a poor inhibitor,[24,25] was found to behave as an uncompetitive inhibitor (Figure 1 panel C). A summary of the kinetic parameters related to the above mentioned inhibitors is reported in Table 2.

When the ability of the adenosine analogues to inactivate SAHase was examined in the hydrolytic direction, the enzyme was incubated with the drug, at selected concentrations. Figure 2A shows the time-dependent inactivation pattern of SAHase upon incubation with 2-ClAdo. At a concentration of 200 μM the drug caused a strong decrease of activity and almost complete inactivation was detectable after 30 min. The enzymatic loss was not accompanied by adenine release from 2-ClAdo (data not shown) thus confirming the observations previously reported by Kim *et al.*[18] A similar, even if weaker effect was found when SAHase was treated with tubercidin under the same conditions described for 2-ClAdo (Figure 2B). Although tubercidin is reported as a potent antiviral agent with a broad spectrum activity, it exhibits little, if any, selectivity in its antiviral action.[26] It was necessary to increase the concentration of this drug up to 400 μM in order to obtain 50% inactivation after 30 minutes incubation with SAHase.

Two other adenosine analogues were assayed only in the synthetic direction because one of them, inosine, did not exert any effect on SAHase monitored in the hydrolytic direction and the other, 2′-deoxyadenosine, is a substrate for adenosine deaminase.

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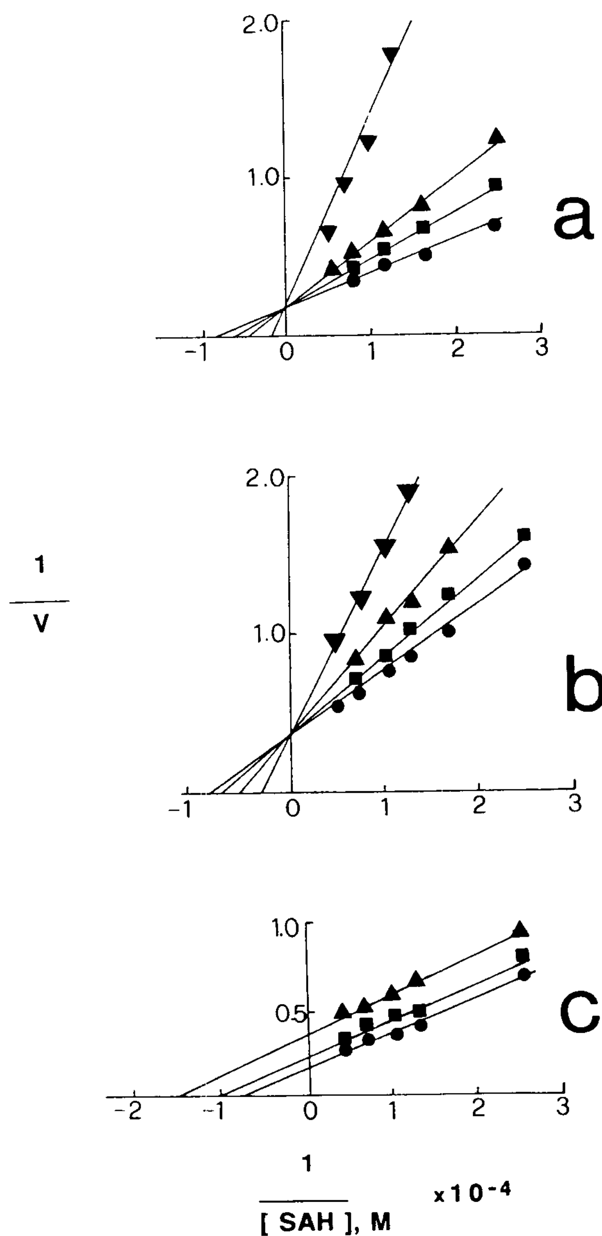

Figure 1 Effects of DZA, DZAry and Nebularine on SAHase activity assayed in the hydrolytic direction. 3 μg of SAHase were incubated with the selected chemical compounds. The reaction was started by adding an excess of ADA 2(U), and monitoring the absorbance decrease at 265 nm. (a) untreated SAHase (●): plus DZA 1×10^{-6} M (■), 5×10^{-6} M (▲), 1×10^{-5} M (▼); (b) control (●): plus DZAry 5×10^{-8} M (■), 1×10^{-7} M (▲), 2×10^{-7} M (▼); (c) control (●): plus Nebularine 1×10^{-4} M (■), 2×10^{-4} M (▲).

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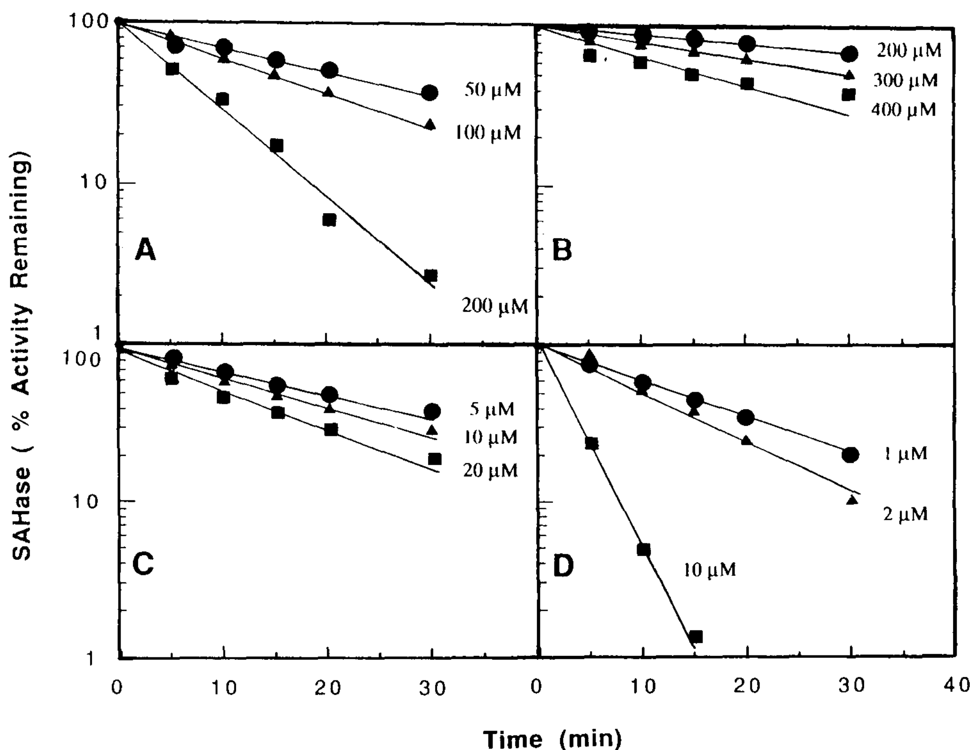

Figure 2 Time-dependent inactivation curves of SAHase treated with 2-Chloroadenosine, Tubercidin, Inosine and 2′-deoxyadenosine. 3 μg of SAHase were incubated with the selected concentrations of 2-ClAdo (A), Tubercidin (B), Inosine (C) and 2′-dAdo (D) at 37 °C in 0.1 M Hepes pH 7.2, 1 mM EDTAm 1 mM DTT in a total volume of 0.1 ml. At the indicated times, 10 μl samples were added to the reaction mixtures containing 50 μM SAH and 2U ADA, and monitored at 265 nm.

As far as 2′-deoxyadenosine is concerned (Figure 2D), a concentration of 10 μM was able to induce an almost complete inactivation of SAHase after 10 min incubation with a linear, rapid decay in activity. Inosine (Figure 2C) showed a slower time-dependent inactivation with a profile quite similar to that found for 2′-deoxyadenosine. It should be noted that inosine is not a substrate of SAHase for the synthesis of inosylhomocysteine adduct; the observed effect, therefore, cannot be attributed to a preferential condensation of the inosinic ring with the L-homocysteine moiety.

It has been previously reported[27] that adenosine deaminase deficiency associated with severe combined immunodeficiency is accompanied by a virtual absence of erythrocytes SAHase activity. Suicide inactivation of SAHase by 2′-deoxyadenosine that accumulates in adenosine deaminase deficiency may account for lack of this secondary enzyme.

Table 3 summarises the adenosine analogues assayed which shows 2′-d-Ado as the most powerful inactivator. To assess the irreversible inactivation of SAHase, the incubated mixtures were extensively dialysed against phosphate buffer and again tested for SAHase activity. The original activity was not restored by this treatment.

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Table 3 K_i values of different inactivators assayed on SAHase

SAHase inactivators	K_I (M)
2′-dAdo	8.9×10^{-6}
Inosine	94.0×10^{-6}
2-Cl-Ado	28.0×10^{-5}
Tubercidin	68.0×10^{-4}

DISCUSSION

Differences in the primary structure of SAHase (such as those observed in the electrophoretic variants of the enzyme from human erythrocytes[25]) are most likely responsible for the different kinetic behaviour observed in the presence of nucleoside analogue inhibitors. Therefore, it appeared of interest to assess whether molecules previously employed as inhibitors and/or inactivators of SAHase purified from different sources,[24] exerted similar effects on the bovine pancreatic enzyme. DZA and DZAry are two of the most potent competitive inhibitors of SAHase found so far in organisms other than man, with K_i values ranging from 10^{-8} to 10^{-9} M. Our results confirm the higher potency of DZAry as compared to DZA. These adenosine analogues mainly involved in the inhibition of transmethylating reactions and of adenosylmethionine decarboxylase, were found to behave as competitive inhibitors, while nebularine displayed an uncompetitive inhibition. Such behaviour was previously observed[25] for three human erythrocyte SAHase variants, in which nebularine exerted a different type of inhibition against each of the three variants, one of them, the less abundantly expressed (type, 3–1) showed a mixed- type inhibition. When bovine pancreas SAHase was incubated with 2-ClAdo or with tubercidin, a time-dependent inactivation appeared linked strictly to the presence of the adenine moiety, since the guanosine and pyrimidine analogues were ineffective (data not shown).

It has been suggested[28] that SAHase binds some nucleosides mainly through hydrophobic interactions. If this is true, the mechanism responsible for the inactivation of SAHase by some of the necleosides described here may not be due to a hindrance of the active site by the compound tested, but rather to binding to cleft(s) other than the adenosine-binding site at the catalytic center. When the effect of the analogues was studied by monitoring the SAHase activity in the synthetic direction, the most effective inactivator was found to be 2′-dAdo, while tubercidin was far weaker. The results reported here are in line with the mechanism previously proposed[29] for the inactivation of SAHase by 2′-dAdo which consists in the oxidation of 2′-dAdo by enzyme-bound NAD^+ and subsequent elimination of adenine.

Our results showed that the multitude of inhibitors and/or inactivators assayed on bovine pancreas SAHase exerted a kinetic behaviour similar to those observed for the enzyme from other sources with the exception of nebularine which displayed an uncompetitive inhibition pattern. It seems that nebularine therefore, among the SAHase inhibitors so far studied, is the only compound showing a typical kinetic behaviour depending on the source of SAHase.

A partial or even a complete inactivation of this enzyme should result in accumulation of SAH, with subsequent inhibition of S-adenosylmethionine-dependent

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methylation, which would presumably interfere with normal cell function in a number of ways. Suicide inactivation of SAHase may have relevance to clinical medicine[18,30–31] in at least two situations. The first is the immune deficiency disease associated with absence of adenosine deaminase activity which is thought to result at least in part from the effects of dATP derived from abnormal accumultion of 2′-dAdo.[32–34] However, such accumulation could also lead to intracellular inactivation of SAHase. The second situation in which one might expect to see inactivation of SAHase is in patients treated with adenosine analogues i.e. 9β-D-arabinofuranosyladenine (Ara-A) for viral infections or for neoplastic diseases. It is reasonable to postulate that the antiviral effects of these compounds are in part attributable to suicide inactivation of SAHase with subsequent accumulation of SAH, given the well documented anti-viral properties of analogues of SAH. Nevertheless, the nucleoside triphosphate derivatives of 2′-deoxyadenosine are also toxic to cells, and it may be difficult to determine to what extent suicide inactivation of SAHase, inhibition of ribonucleotide reductase, and in some cases (Ara-ATP) inhibitors of DNA polymerase activity, contribute to phenomena as complex as immune deficiency or inhibition of viral infection.[35–37] In conclusion, the selective inhibition, or even more, the suicide inactivation of SAHase, with subsequent inhibition of methyl-transfer reactions, may be a potentially useful approach to the chemotherapy of viral infections and neoplastic diseases in which SAHase activity has a relevant role in the maintenance of diseases. Based on studies of the structural features of inactivating nucleosides, it may be possible to design chemical compounds which specifically employ this mechanism.

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