Pergamon

Biochemical Pharmacology, Vol. 48, No. 5, pp. 897–903, 1994.
Elsevier Science Ltd.
Printed in Great Britain
0006-2952/94 \$7.00+0.00

0006-2952(94)00247-9

EFFECTS OF NOVEL ANTI-VIRAL ADENOSINE ANALOGUES ON THE ACTIVITY OF *S*-ADENOSYLHOMOCYSTEINE HYDROLASE FROM HUMAN LIVER

KRYSTYNA FABIANOWSKA-MAJEWSKA,* JOHN A. DULEY† and H. ANNE SIMMONDS
Purine Research Laboratory, Clinical Science Laboratories, UMDS Guy's Hospital,
London SE1 9RT, U.K.

(*Received* 18 *January* 1994; *accepted* 16 *May* 1994)

Abstract—Some adenosine analogues have been found previously to inhibit *S*-adenosylhomocysteine (SAH)-hydrolase activity in erythrocyte lysates. In this study, the enzyme was purified 500-fold from human liver and its M_r found to be 190,000. Its kinetics in the synthase direction were studied, the K_m for adenosine being determined as 32 μM. Several purine nucleoside analogues currently used in anti-tumour and antiviral therapy were tested for their influence on SAH-hydrolase activity. The results confirmed our previous findings for the unpurified human erythrocyte enzyme, and demonstrated that the most potent inhibitors of human liver SAH-hydrolase were neplanocin A, 7-deaza-adenosine (tubercidin), 2′-deoxyadenosine, and 9-β-D-arabino-furanosyladenine. Analogues showing intermediate inhibition were 2′3′-dideoxyadenosine (2′3′-ddAdo), 5′-deoxy-5′-methyl-thioadenosine, 3′-deoxy-adenosine, 2-chloroadenosine, 1,2,4-triazole-3-carboxamide riboside (ribavirin), δ-adenosylornithine (sinefungin), *S*-adenosylmethionine, 5-amino-4-imidazole carboxamide riboside (AICAR), and 5′-iodo-5′-deoxyadenosine (5′I,5′-dAdo). Weak or no inhibition was noted with 5′-deoxyadenosine, 5-hydroxyimidazole-4-carboxamideriboside (bredinin), inosine and its deoxy analogues, and acyclovir. Our results show that drugs such as 2′3′-dideoxyadenosine (used in HIV therapy) and ribavirin (an inhibitor of inosinate dehydrogenase), in addition to their other known mechanisms of action, have an inhibitory effect on SAH-hydrolase activity, which may be of significance in their antiviral action.

Key words: *S*-adenosylhomocysteine hydrolase; ribavirin; neplanocin A; dideoxyadenosine; antiviral drugs; HIV

SAH‡-hydrolase (EC 3.3.1.1) is considered to be one of three enzymes involved in the "active methyl" cycle, and perturbation of *S*-adenosylmethionine-dependent transmethylation reactions can occur following inhibition of this enzyme. For this reason, SAH-hydrolase has became a pharmacological target for the development of new chemotherapeutic agents.

The mechanisms of toxicity of 2′-dAdo, which accumulates in subjects with immunodeficiency due to inherited adenosine deaminase deficiency, have been studied widely and include inhibition of ribonucleotide reductase, the enzyme responsible for formation of the normal pool of deoxynucleotides required for DNA synthesis [1]. However, the toxic effect of 2′-dAdo is also significant in cells which lack ribonucleotide reductase activity, and this may relate to the fact that 2′-dAdo causes inactivation of SAH-hydrolase and consequently perturbation of *S*-adenosylmethionine-dependent transmethylation reactions [2].

Recently, other purine and deoxypurine analogues have been introduced into antiviral therapy. Thus 2′3′-ddAdo, 2′3′-ddIno, ribavirin and combinations of these antimetabolites have been used with, for example, other SAH-hydrolase inhibitors [3–6]. Currently, knowledge of the bioactive mechanisms of purine and pyrimidine nucleoside analogues is poor and their potential effects on SAH-hydrolase activity, leading to inhibition of methylation reactions in which *S*-adenosylmethionine is the methyl donor, have been ignored [3, 7–9].

Our preliminary studies noted an inhibitory effect of several purine nucleoside analogues on SAH-hydrolase activity in erythrocyte lysates [10]. The present studies have extended the number of novel analogues tested, and examined their inhibitory effects on the activity of SAH-hydrolase isolated from human liver. These investigations show that the molecular mechanisms of toxicity of these drugs may relate not only to their metabolism to nucleotide derivatives as currently believed, but also to their effect on the "active methyl" cycle *via* its key enzyme, SAH-hydrolase.

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* On leave from Department of General Chemistry, Medical University of Lodz, Lodz, Poland.

† Corresponding author. Tel. (0)71-955 40 24; FAX (0)71-407 66 89.

‡ Abbreviations: SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; MTA, 5′-deoxy,5′-methylthioadenosine; 2′-dAdo, 2′-deoxyadenosine; 2′3′-ddAdo, 2′3′-dideoxyadenosine; 2′3′-ddIno, 2′3′-dideoxyinosine; AraA, 9-β-D-arabinofuranosyladenine; ribavirin, 1,2,4,triazole-3-carboxamide riboside; bredinin, 5-hydroxyimidazole-4-carboxamide riboside; tubercidin, 7-deazaadenosine; 5′I,5′-dAdo, 5′-iodo-5′-deoxy-adenosine; acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanine; sinefungin, adenosylornithine; AICAR, 5-amino-4-imidazole carboxamide riboside; Ino, inosine; 2′-dIno, 2′-deoxyinosine; 2-ClAdo, 2-chloroadenosine.

Table 1.

Purification step	Total protein (mg)	Protein (mg/mL)	Specific activity (U/mg of protein)	Purification factor
1. Crude homogenate	8640	120	0.0013	1.0
2. Cytosol (100,000 *g*)	2525	52.5	0.0029	2.3
3. Ammonium sulfate (0.35–0.5 sat)	680	36.5	0.0050	4.0
4. DEAE Sephacel	112	17.2	0.0945	75.0
5. Sephacryl S-300	8.4	0.7	0.6150	448.0

MATERIALS AND METHODS

Purification procedure. SAH-hydrolase was partially purified from the cytosolic fraction of human liver. For this purpose, normal human liver was obtained post-operatively as excess tissue cut down for transplantation, and was frozen at −70° until use. Subsequent enzyme isolation was performed at 4°. A liver portion (approximately 10 g) was minced and homogenised in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol (buffer A). The crude homogenate was centrifuged at 15,000 *g* for 20 min, then the supernatant was centrifuged again at 100,000 *g* for 60 min. The resulting cytosolic fraction was brought to 35% saturation with ammonium sulfate by adding the solid, and the precipitate discarded. Solid ammonium sulfate was added to the supernatant to 50% saturation, and the resultant precipitate was dissolved in 5 mL of buffer A, and exhaustively dialysed for 40 hr against the same buffer A.

The dialysate was then applied to a DEAE Sephacel column previously equilibrated with buffer A, and the enzyme was eluted by buffer A with a linear gradient of KCl (to 0.2 M). Fractions with the highest SAH-hydrolase activity were collected, then concentrated in a dialysis sac using "Aquacide" (Calbiochem, U.K.), and applied to a second column, Sephacryl S-300, which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol (buffer B). The column was then eluted isocratically with buffer B. Fractions exhibiting high SAH-hydrolase activity were pooled for subsequent experiments.

The M_r of SAH-hydrolase was determined by Sephacryl G-150 gel filtration according to Andrews [11]. Bovine serum albumin (M_r 67,000), apoferritin (M_r 440,000) and yeast alcohol dehydrogenase (M_r 150,000) were used as standards. The K_m value for adenosine was estimated from six substrate concentrations using the Harvard Graphics plotting programme for both Hanes and Lineweaver–Burk plots. Protein was measured by Sedmak's method with Coomassie brilliant blue reagent [12].

Enzyme assays. SAH-hydrolase activity was assayed in the "synthase" direction. The standard incubation mixture in a final volume of 340 μL was as previously published [10], using both non-labelled and [8-^{14}C]adenosine (0.5 mM). The various adenosine analogues were tested at double the concentration (1 mM) of adenosine in the incubation mixture. Conversion of adenosine into SAH was determined by HPLC using a 5 μ ODS-2 column (12.5 × 0.45 cm) (Hichrom, U.K.). The product was separated from substrate and inhibitors by isocratic elution with phosphate buffer (1 g/L KH_2PO_4), adjusted to pH 4.2 with KOH, containing 9% v/v methanol. MTA phosphorylase was assayed as described previously [13], and the adenine released following incubation was analysed by HPLC using the same column as for the SAH-hydrolase assay, but with isocratic elution with phosphate buffer, pH 4.2, and 20% methanol. Adenosine deaminase and purine nucleoside phosphorylase were assayed utilising the HPLC method of Fairbanks *et al.* [14].

Chemicals. D,L-homocysteine, MTA, [8-^{14}C]-adenosine, and most of the adenosine analogues were obtained from Sigma Chemical Co. (U.K.). Neplanocin A and bredinin were the generous gifts of Dr T. Saito (Toyo Yozo Co. Ltd, Japan), and ribavirin of ICN Pharmaceuticals (U.K.). Sephacryl S-300 and DEAE Sephacel were obtained from Pharmacia Fine Chemicals (U.K.).

RESULTS

Physicochemical characterisation of partially purified human liver SAH-hydrolase

All the purification steps are summarised in Table 1. Fractions with the highest activity of SAH-hydrolase but containing no detectable adenosine deaminase, purine nucleoside phosphorylase, or particularly MTA-phosphorylase, were used for determination of the kinetic properties. The specific activity of the isolated human liver SAH-hydrolase in the synthase direction was calculated to be 0.615 U/mg protein (488-fold purification). The assay was linear with respect to time and enzyme concentration up to approximately 53% conversion of adenosine to SAH under the conditions described in Materials and Methods.

At saturating D,L-homocysteine concentrations (4.5 mM) the apparent K_m value (mean of four experiments) estimated from a Hanes plot was 32 ± 3 μM for adenosine (Fig. 1). Estimation by Lineweaver–Burk plot gave the same result. The M_r of the enzyme purified approximately 500-fold was estimated by Sephadex gel filtration to be 190,000.

Inhibition of SAH-hydrolase activity by natural and synthetic purine analogues

Analogues were tested using the SAH-hydrolase purified from human liver. Figure 2 shows the

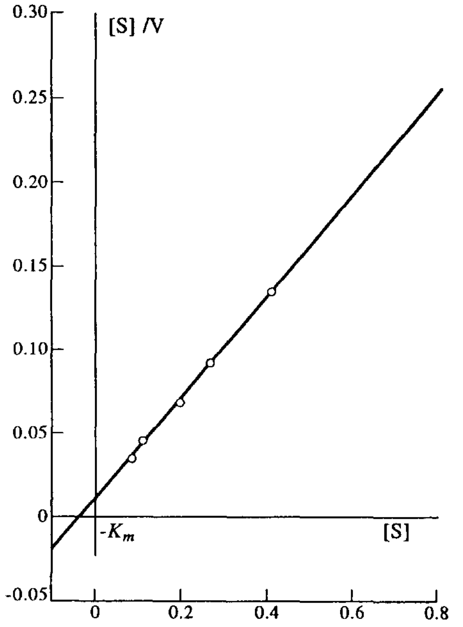

Fig. 1. Determination of the adenosine K_m value for human liver SAH-hydrolase by a Hanes plot. The substrate concentration [S] is shown as mM, and yielded a K_m value of 0.032 mM at the abscissa intersect. Expression of the data as a Lineweaver–Burk plot gave the same K_m.

structural formulae of some of these analogues, arranged in four groups according to their structural similarities: the first group comprised adenosine and the cyclopentene analogue neplanocin-A; the second group had 2′ or 3′-deoxyadenosine substitutions of the ribose; the third group were analogues of AICAR, having a broken pyrimidine ring on the purine component; and the fourth group comprised 5′-hydroxy substitutions of adenosine, including 5′-deoxyadenosine. The effect of substitution of the adenine moiety, was tested using 2-chloro-adenosine and 7-deaza-adenosine (tubercidin). In addition, inosine and some analogues were assessed. The results of all experiments are summarised in Fig. 3.

Complete inhibition of the hydrolase was obtained with extremely low concentrations of neplanocin-A (2–5 nM), a tight binding inhibitor [15]. In addition, the 2′- and 3′-deoxyadenosine analogues in the second group were potent inhibitors of the enzyme. The results showed that for this group, inhibition decreased as follows: 2′-dAdo > 2′3′-ddAdo > 3′-dAdo (99%, 80% and 70% inhibition, respectively), while AraA caused 98% inactivation of the isolated enzyme. Substitution in the adenine moiety also resulted in complete inhibition in the case of tubercidin, while 2-ClAdo strongly inhibited (70%) SAH-hydrolase.

The third group comprised AICAR, which is an intermediate of *de novo* purine synthesis, and its analogues. AICAR resulted in 40% inhibition of SAH-hydrolase, while ribavirin, a potent antiviral inhibitor of IMP dehydrogenase, caused 66% inhibition by a non-competitive mechanism (K_i of 2.5×10^{-4} M). In contrast, bredinin, a 5-hydroxyl analogue of AICAR, showed negligible inhibition.

The fourth group comprised 5′-ribose-substituted adenosine analogues. MTA, an intermediate of the polyamine pathway, strongly inhibited the hydrolase activity by 80%, while SAM, the active substrate of the cellular methylation cycle, inhibited it by 55%, and sinefungin, a SAM analogue, caused 65% inhibition. Also in this group, 5′I,5′-dAdo resulted in an intermediate reduction in activity of about 40%, but 5′-dAdo had only weak inhibition, of 20%. Inosine and its deoxy analogues were also tested, but resulted in negligible inhibition.

DISCUSSION

The results presented here, demonstrating SAH-hydrolase as a potential target for many chemotherapeutic adenosine analogues, suggest that this enzyme plays an important role in the action of novel antiviral and antitumour drugs which are also adenosine analogues. Although the mechanism of toxicity of most adenosine analogues appears multifactorial, these *in vitro* studies are the first in which an attempt has been made to reappraise these effects and, in particular, to implicate SAH-hydrolase in the cellular toxicity of 2′3′-ddAdo and ribavirin.

The rationale for the SAH-hydrolase purification was not the production of an homogeneous protein, but to produce an enzyme lacking any possible contamination by other enzymes which might metabolise adenosine or its analogues, namely MTA-phosphorylase, adenosine deaminase, and purine nucleoside phosphorylase. Adenosine kinase was not considered a problem as its substrate ATP was absent from the assays.

The M_r of 190,000 for SAH-hydrolase, purified approximately 500-fold, was in agreement with values for the enzyme from other mammalian tissues [16]. By contrast, the K_m values reported for adenosine as substrate have been controversial, ranging from 0.2 μM to 420 μM [16]. In this study, the K_m of 32 μM was higher compared with the K_m obtained for horse liver (0.49 μM) [2], rat liver (0.6 μM) [17] or human placenta (0.9 μM) [18]. These inconsistencies might be explained by modification of the enzyme during isolation, or the presence of some factors which are critical for the kinetic behaviour of the enzyme *in vitro*, as previously reported by Ueland [16], or alternatively by species as well as tissue differences due to isoenzymes of the hydrolase.

This study focused mainly on the effect on isolated SAH-hydrolase activity of adenosine analogues which could be broadly classified into groups according to the structural formulae (Fig. 2). The first group contained only the adenosine analogue neplanocin A, which may be a substrate for adenosine deaminase and adenosine kinase. It has also been noted that *S*-neplanocinylmethionine is formed in parasitised red cell cultures, which indicates a SAH-hydrolase-mediated role [15, 19].

The high affinity of the hydrolase for neplanocin, with a K_i of 10^{-9} M, is in accordance with the data for this cytotoxic drug, which has been used in anti-tumour therapy [19]. The drug is known to cause a

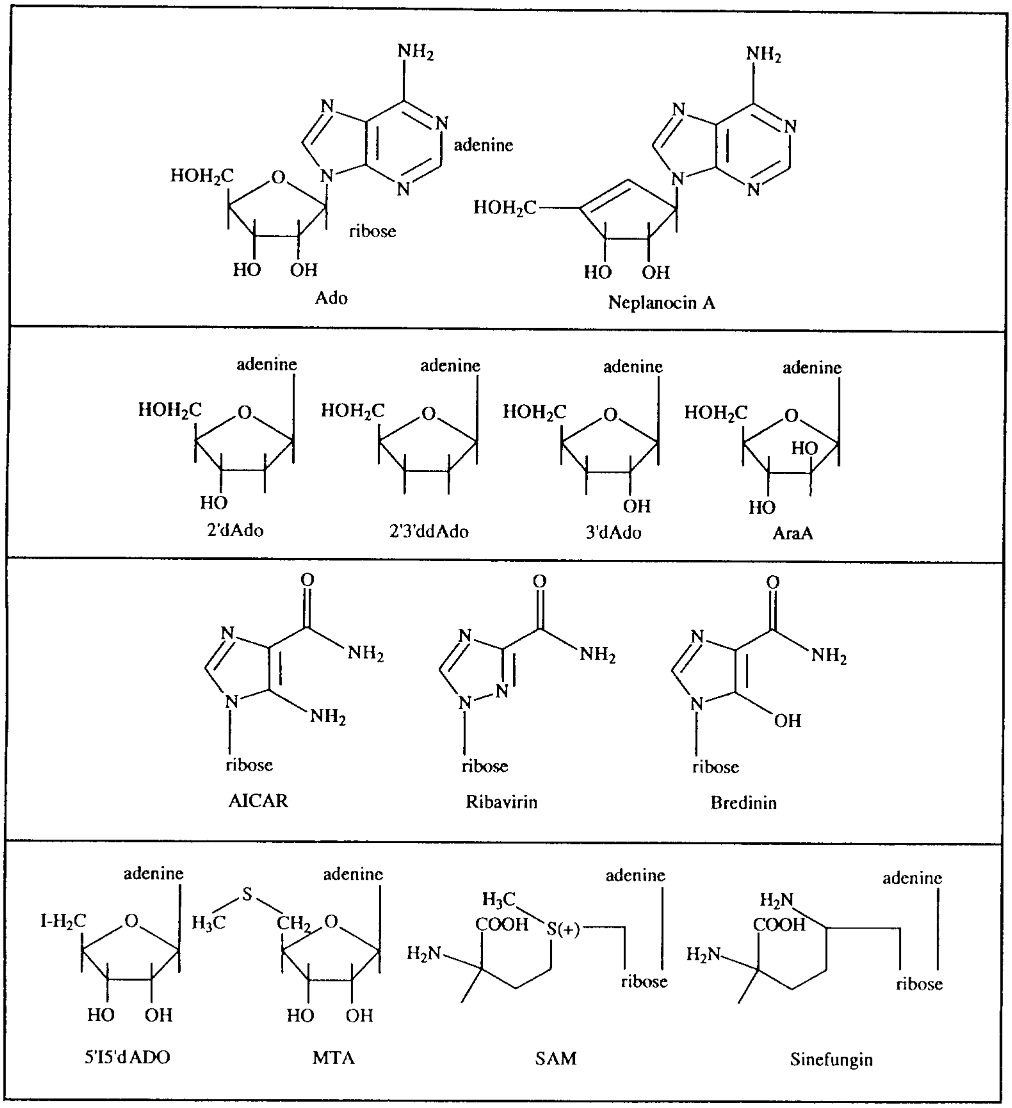

Fig. 2. Structural formulae of analogues tested. These have been grouped according to structural similarities, starting from the top: (1) adenosine and Neplanocin; (2) 2'- and 3'-deoxyadenosines; (3) AICAR and drug analogues; (4) analogues substituted at the ribose 5' position; this group includes 5'-deoxyadenosine (not shown). Also not shown are 2-chloroadenosine and 7-deaza-adenosine (tubercidin), both containing substituted adenines.

rise in the concentrations of SAM and SAH in red blood cells, confirming an inhibitory effect on SAH-hydrolase activity [7, 15].

The second group of analogues comprised the following compounds: 2'- and 3'-deoxyribose analogues, including AraA. Although the presence of an hydroxyl group in the 3' position of the sugar ring increased the inhibitory effect of adenosine analogues, the compounds with 3'-deoxyribose showed only a slightly reduced inhibitory effect, and it has already been reported that 3'-dAdo (cordycepine) is an inhibitor of SAH-hydrolase *in vitro* [16]. The toxicity of 2'-dAdo has been widely discussed and explained [2, 16, 20, 21]. The results of experiments for AraA were placed together with 2'-dAdo analogues, because of both its ability to inactivate the isolated enzyme and its probable similar mechanism of toxicity to 2'-dAdo [7, 16, 21, 22].

The metabolism and mechanisms of toxicity of 2'3'-ddAdo are still being elucidated, and it has been reported that this drug can be rapidly metabolised by adenosine deaminase to 2'3'-ddIno, and by deoxycytidine kinase to adenine dideoxynucleotides [3, 9, 23]. In the latter case, 2'3'-ddATP has been shown to act as both an inhibitor and substrate of HIV reverse transcriptase [6]. Thus the potent inhibitory effect of 2'3'-ddAdo on SAH-hydrolase from human liver demonstrated here may be significant, as it exposes a hitherto unknown biochemical mechanism of action of this drug. It is thus probable that *in vivo*, the cytotoxicity of 2'3'-ddAdo relates at least partly to inactivation of SAH-hydrolase.

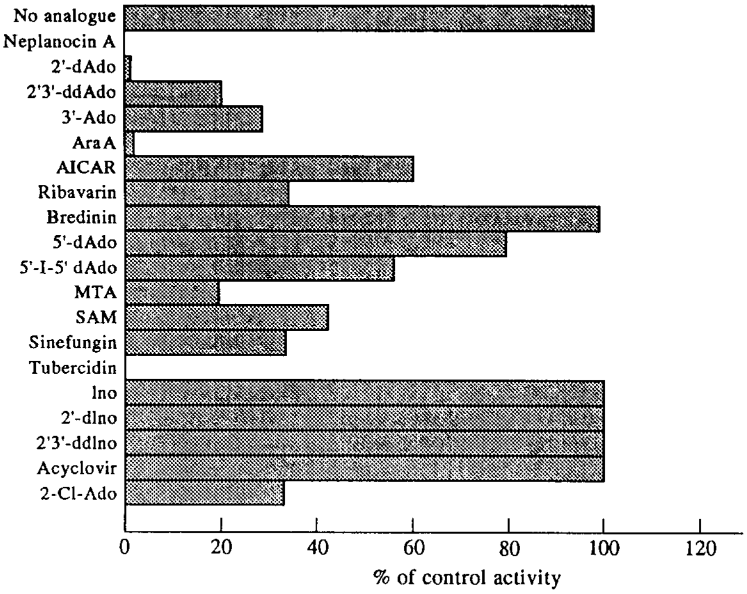

Fig. 3. Inhibition of human liver SAH-hydrolase activity by adenosine analogues. Enzyme activity in the presence of the analogues listed (1 mM), calculated as a percentage of activity with substrate (0.5 mM adenosine) only. Results are the average of at least four experiments. The differences between separate experiments were less than 15%.

Analogues containing a broken pyrimidine ring on the purine moiety, i.e. AICAR and its analogues ribavirin and bredinin, formed the third group of inhibitors tested. The result with ribavirin is noteworthy because from published data it is known only that the drug is metabolised to the triphosphate derivative, which is considered primarily responsible for its antiviral action [7, 8, 24]: the effect by this drug on SAH-hydrolase activity has not been reported.

Since it has been proposed that ribavirin inhibits 5′-capping of mRNA, it seems possible that the inactivation of hydrolase activity by ribavirin may result in the perturbation of the SAM-dependent trans-methylation reaction, which is the final step of the above-mentioned 5′-capping reaction. Furthermore, it has been noted that the therapeutic effect of ribavirin is enhanced when administered in combination with 2′3′-ddAdo, whose effect on SAH-hydrolase activity is described above. The biochemical basis for the synergistic action of ribavirin and 2′3′-ddAdo has not been elucidated previously [6].

The inhibitory action of AICAR analogues on SAH-hydrolase was lost in the case of bredinin, whose hydroxyl group replaces the amino group of AICAR. This suggests that a nucleophilic group is essential near the 3-position of the pyrimidine ring, for the hydrolase to bind [25].

In contrast to this, substitution of the complete adenine ring had a more powerful effect on the hydrolase. Thus, tubercidin completely inhibited the enzyme, which may account in part for its wide spectrum of cytotoxicity which has resulted in its use for antifungal, antitubercular and antineoplastic therapy [26]. This drug is also a substrate for adenosine kinase, readily forming ATP analogues. Another adenine-substituted drug, 2-chloro-adenosine [10, 27], inhibited SAH-hydrolase by 65%.

A separate group was composed of the adenosine analogues substituted at the 5′-position of the ribose. The inhibition by MTA of human liver SAH-hydrolase demonstrated in this paper confirms previous reports of the potent effect of this and similar analogues on SAH-hydrolase activity [16, 28, 29], and agrees also with our previous studies with the hydrolase from human erythrocyte lysates [10]. Our experiments with human liver SAH-hydrolase also included determination of inhibition by SAM, the substrate for transmethylation reactions, and sinefungin, an antibiotic isolated from *Streptomyces griseolus* which is a potent inhibitor *in vitro* of some transmethylases [16]. Both reagents inhibited SAH-hydrolase.

Where a simple 5′-deoxy substitution of the ribose occurred, hydrolase affinity and inhibition was poor. It is worth noting that 5′-deoxy analogues of adenosine, apart from sinefungin and SAM, are substrates for MTA-phosphorylase (results not shown). In contrast, none of this group is metabolised by adenosine deaminase [30].

Purines with no effect on isolated SAH-hydrolase included Ino, 2′-deoxy-Ino, 2′3′-ddIno and acycloguanosine (acyclovir, a potent antiviral). These results for inosine and its deoxy analogues are not in agreement with data in which 40% inactivation of human placenta SAH-hydrolase was reported, and in which *S*-inosylhomocysteine synthesis was simultaneously detected [16, 31]. This supports the concept that there may be tissue-specific isozymes.

These studies have enabled quantitative estimations of the comparative inhibitory effects of nucleoside analogues, and have related these effects to their structural differences. It is likely that the demonstrated effect of these drugs *in vitro* on SAH-hydrolase activity extends also to *in vivo* inhibition, with consequent disruption of the activity of "active methyl" cycle pathways, which can have far-reaching consequences.

Acknowledgements—One of us (K. F.-M.) gratefully acknowledges the support of a Fellowship Award from the Wellcome Trust. We would like to thank the Special Trustees of Guy's Hospital for support, and Dr Portman of the Liver Unit of King's Hospital, London, for kindly supplying the human liver samples.

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