From: Biological Methylation and Drug Design,
Edited by Ronald T. Borchardt, Cyrus R. Creveling, and Per Magne Ueland.
The Humana Press, 1986.

DISPOSITION OF ENDOGENOUS S-ADENOSYLHOMOCYSTEINE AND HOMOCYSTEINE FOLLOWING EXPOSURE TO NUCLEOSIDE ANALOGUES AND METHOTREXATE

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INTRODUCTION

S-Adenosylhomocysteine (AdoHcy) is a product from and an inhibitor of S-adenosylmethionine-dependent transmethylation reactions. This compound is degraded to adenosine and L-homocysteine (Hcy) in the cell through the action of the enzyme AdoHcy hydrolase (EC 3.3.1.1.) (de la Haba & Cantoni, 1959). The view is held that the AdoHcy hydrolase reaction is the only source of Hcy in vertebrates (Cantoni & Chiang, 1980). Hcy is converted to cystathionine or is salvaged to methionine. In all tissues, except liver and kidney, the latter pathway is catalyzed by a single enzyme, which requires 5-methyl - tetrahydrofolate as methyl donor (Mudd & Levy, 1983). These metabolic relations are depicted in figure 1.

Interest in AdoHcy metabolism was stimulated by the pioneering work of Chiang et al. (1977) and Hershfield (1979) demonstrating that AdoHcy hydrolase is inhibited by various adenosine analogues and some analogues serve as substrate for this enzyme. Consequently, these adenosine analogues may induce high level of intracellular

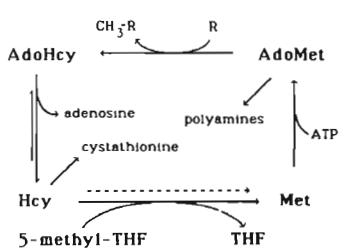

FIGURE 1: Metabolism of AdoHcy, Hcy and related compounds. THF, tetrahydrofolate.

nucleosidylhomocysteine, which in turn block AdoMet dependent methyltransfer reactions (for review, see Borchardt, 1980; Ueland, 1982). These observations form the basis for the concept of AdoHcy hydrolase as a target enzyme for adenosine analogues.

The first part of this article reviews data from our and other laboratories, on the effect of nucleoside analogues on AdoHcy catabolism *in vivo*. Because the biological consequences of AdoHcy accumulation have been thoroughly debated (Ueland, 1982), we have recently focused on the pharmacological perturbation of Hcy metabolism. This may be obtained by inhibitors of AdoHcy catabolism, but also by antifolate drugs leading to deprivation of reduced folates. Some recent data on this topic are presented.

INHIBITION OF ADOHCY CATABOLISM IN VIVO

Numerous nucleoside analogues function as inhibitors, inactivators (irreversible inhibitors) or substrates of isolated AdoHcy hydrolase (Ueland, 1982). However, effectiveness in cell free system does not ensure potent inhibition of AdoHcy catabolism in the intact cell. This is clearly demonstrated with 2-chloroadenosine and aristeromycin which are among the most effective inhibitors of AdoHcy hydrolase yet discovered (Chiang et al., 1981), but which have only a slight inhibitory effect on AdoHcy degradation in intact cells (Schanche et al., 1984b). Factors which may counteract the intracellular effect of an inhibitor of AdoHcy hydrolase, include slow transport, metabolic degradation, protection of the enzyme against inactivation by natural substrates and reactivation of inactivated enzyme (Schanche et al., 1984a,b). In addition, there may be species differences with respect to the response of AdoHcy hydrolase to a particular nucleoside analogue, as has been demonstrated for 3-deaza - adenosine (Kim et al., 1983) and neplanocin A (Borchardt et al., 1984; Glazer et al, 1984).

Some nucleosides, which are potent inhibitors of AdoHcy catabolism in intact cells, are listed in Table 1. Some properties assumed to be critical for their effectiveness *in vivo,* are also shown.

High affinity towards the target enzyme, AdoHcy hydrolase, and metabolic stability of the nucleoside analogue are factors which seem critical for inhibition of AdoHcy catabolism *in vivo*. This statement is supported by data listed in table 1. Active compounds include adenosine analogues modified in the purine and/or the sugar moiety. Substitution of the nitrogen in the 3 position of the purine skeleton with carbon seems to be well tolerated by the adenosine binding site of AdoHcy hydrolase, but the 3-deaza-analogues are not substrate of other adenosine metabolizing enzymes (Montgomery et al., 1982; Schanche et al., 1984b). Several active compounds modified in the ribose moiety are acyclic adenosine derivatives. These include D-eritadenine (Votruba & Holy, 1982;

Table 1

Some properties of nucleoside analogues acting as potent inhibitors of AdoHcy catabolism in vivo [a]

Compound	Mode of action	Substrate of AK	Substrate of ADA	Effect on cellular AdoHcy
c^3Ado	Substrate and inhibitor (K_i=4μM)	NS	NS	marked increase
c^3ara-A	Inhibitor	NS	NS	moderate increase
c^3Ari	Inhibitor (K_i=1nM,4nM, 3μM)	NS	NS	marked increase
ara-A	Inactivator and inhibitor (K_i= 5μM)	S	S	marked increase
D-Erit-A	Inactivator and inhibitor (IC_{50}=7nM)	NS	NS	marked increase
Neplanocin A	Inactivator and inhibitor (K_i= 8 nM)	S	S	marked increase*
Ado-ox	Inactivator and inhibitor (K_i= 3nM)	S	S	marked increase

[a]Data taken from or quoted in the following articles: Patel-Thombre & Borchardt, 1985; Schanche et al.,1984a,b; Ueland, 1982; Votruba & Holy, 1982.
c^3Ado, 3-deazaadenosine; c^3ara-A, 3-deazaadenine arabinoside; c^3-Ari, 3-deazaaristeromycin; ara-A, adenine arabinoside; D-Erit-A, D-eritadenine; Ado-ox, periodate oxidized adenosine; ADA, adenosine deaminase; AK, adenosine kinase; NS, not substrate; S, substrate;
*Inhibits AdoHcy catabolism in some cells (Borchardt et al., 1984).

Schanche et al., 1984a), adenosine dialdehyde (Hoffman, 1980; Bartel & Borchardt, 1984) and related compounds (Houston et al., 1985).

DISPOSITION OF ADOHCY IN CELLS EXPOSED TO NUCLEOSIDE ANALOGUES

Treatment of isolated cells or whole animals with adenosine analogues inhibiting AdoHcy hydrolase *in vivo*, leads to a massive accumulation of AdoHcy. This has been demonstrated for all compounds listed in table 1. Even in the presence of high concentrations of nucleoside analogues inactivating AdoHcy hydrolase, a small residual fraction of the intracellular enzyme seems to be protected from inactivation (Helland & Ueland, 1982a). The elevation of AdoHcy may be dependent on the degree of AdoHcy hydrolase inhibition. In addition, the elevation of AdoHcy is largely dependent on cell type or tissue under study. Inhibition of AdoHcy catabolism in whole liver or isolated hepatocytes leads to extremely high levels of AdoHcy, corresponding to a [AdoHcy]/[AdoMet] ratio higher than 1. The increase in AdoHcy in kidney is also pronounced, whereas in most other cell types, the AdoHcy content approaches but does not exceed the amount of AdoMet (Helland & Ueland, 1983). The marked AdoHcy response in liver and kidney is probably explained by the high turnover rate of AdoMet in these tissues (Hoffman, 1981), but may also partly be related to the presence of transmethylation reactions not sensitive to the inhibitory effect of AdoHcy (Helland & Ueland, 1983). The different AdoHcy response in different tissues explains the finding that some AdoMet dependent transmethylation reactions are nearly completely blocked in liver exposed to nucleoside analogues (Hoffman et al., 1980; Schanche et al., 1982), whereas only partial inhibition is observed in some cells (Bartel & Borchardt, 1984).

High intracellular level of AdoHcy induced by nucleoside analogues leads to AdoHcy egress into the extracellular medium. This has been demonstrated for isolated perfused liver (Hoffman et al., 1980), in whole animals (Helland & Ueland, 1983) and with isolated (Helland & Ueland,1982a) and cultured cells (Bartel & Borchardt, 1984; Carson et al.,1982; Helland & Ueland, 1982a). Cells exposed to a potent inhibitor of AdoHcy hydrolase for a few hours may release AdoHcy into the medium in amounts which equal that retained within the cells (Helland & Ueland, 1982a). Thus, a transport mechanism for AdoHcy may exist, which relieves the accumulation of AdoHcy under conditions where its catabolism is inhibited.

HOMOCYSTEINE IN TISSUES AND CELLS

Homocysteine is present in various tissues (Ueland et al., 1984). About 50 % could be extracted with acid and is referred to as free homocysteine whereas a portion is tightly associated with tissue proteins, probably via disulfide linkage. In the liver, free Hcy was localized to the soluble fraction, whereas bound Hcy was about equally distributed

between the soluble and microsomal fractions (unpublished). Liver contained highest the level of Hcy; somewhat lower concentrations were observed in kidney and other tissues (Table 2).

Table 2
Distribution of homocysteine in rat tissues

Tissue	Free Hcy*	Bound Hcy*	Free/Bound*
	(nmol/g)	(nmol/g)	
Liver	4.57±1.09	3.04±0.28	1.47±0.25
Kidney	2.10±0.29	1.73±0.52	1.52±0.32
Spleen	1.62±0.31	0.53±0.02	3.15±0.90
Heart	1.70±0.24	1.10±0.12	1.60±0.20
Lung	1.87±0.18	1.40±0.12	1.36±0.13
Cerebellum	5.15±1.07	0.29±0.03	17.81±2.87
Cerebrum	0.78±0.17	0.33±0.05	2.72±0.70

*Mean of 6 determinations ± S.E.M.

The presence of homocysteine has been demonstrated in isolated hepatocytes (unpublished results), cultured fibroblasts (Ueland et al., 1985) and lymphocytes (German et al., 1983). In the hepatocytes, both free and bound Hcy could be demonstrated in proportions equal to those found in whole liver. Pulse-chase experiments with radioactive methionine labelled in the sulfur atom showed that there was isotope equilibrium between AdoHcy and free and bound Hcy in these cells. This finding is in accordance with the current view (Cantoni & Chiang, 1980) that AdoHcy is the source of Hcy and suggests that a rapid equilibrium exists between free and bound Hcy. When free and bound Hcy are regarded as a single pool, the half-life of Hcy in isolated rat hepatocytes was calculated to about 2 seconds.

Copious amounts of Hcy was released into the extracellular medium from both isolated liver cells and cultured cells, and the Hcy egress was greatly enhanced by addition of methionine to the cellular medium. Thus, the Hcy egress is probably dependent on the metabolic flux through the AdoHcy hydrolase pathway, which in turn is enhanced by the presence of excess methionine.

HOMOCYSTEINE IN TISSUES AND CELLS EXPOSED TO NUCLEOSIDE ANALOGUES

We have investigated the effect of injecting the drug combination ara-A

plus 2'-deoxycoformycin into mice on free Hcy in various organs (Ueland et al., 1984). This treatment almost completely inactivated AdoHcy hydrolase in various tissues, and the amount of AdoHcy increased drastically. Notably, there was no decrease in the Hcy content in tissues, and in kidney a moderate increase in Hcy was in fact observed.

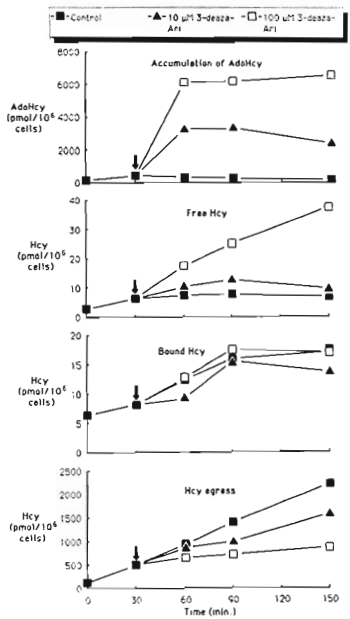

FIGURE 2: Disposition of endogenous Hcy by isolated hepatocytes following inhibition of AdoHcy catabolism by 3-deazaaristeromycin. Isolated rat hepatocytes ($5x10^6$ cells/ml) were preincubated with 200 µM methionine. After 30 minutes, the incubation medium was supplemented with either 10 µM or 100 µM 3-deaza-aristeromycin (arrow). Intracellular AdoHcy (upper panel), free Hcy, protein-bound Hcy and Hcy release into the extracellular medium were determined for the hepatocytes exposed to 3-deaza-aristeromycin and for control cells.

The observation that inhibition of AdoHcy degradation to Hcy is not associated with cellular depletion of Hcy (Ueland et al., 1984) raised several important questions on the source and metabolic fate of cellular Hcy. Answers to these questions were sought by investigating the effect of nucleoside analogues serving as inhibitors of AdoHcy hydrolase on the disposition of endogenous Hcy in isolated rat hepatocytes. The results obtained with 3-deazaaristeromycin are shown in figure 2. This compound is a potent inhibitor of intracellular AdoHcy hydrolase in these cells (Schanche et al., 1984b) and increases the AdoHcy content to extremely high levels in a dose dependent manner. Notably, 3-deaza-aristeromycin slightly increased both free and protein bound Hcy in isolated rat hepatocytes, and this effect was more pronounced in cells accumulating large amounts of AdoHcy. Furthermore, whereas control cells released Hcy into the medium, the Hcy egress was partly blocked in the presence of low levels of 3-deazaaristeromycin, and was almost

totally inhibited by high levels of 3-deazaaristeromycin (Fig. 2). These data are in accordance with those obtained with whole animals injected with the drug combination ara-A plus 2'-deoxycoformycin (Ueland et al., 1984).

The amount of intracellular Hcy seems to be tightly regulated and to be under the influence of the cellular content of AdoHcy. When the production of Hcy is blocked this is compensated for by a reduction or total inhibition of export of Hcy into the extracellular medium. It is conceivable that intracellular Hcy is critical for some vital cellular function, and the Hcy export may be important for maintenance of cellular Hcy within certain limits. The Hcy egress may therefore be a measure of the intracellular balance between Hcy production and utilization.

Kim et al. (1982) have provided indirect evidence that the cytostatic activity of 3-deazaaristeromycin is mediated by inhibition of homocysteine synthesis required for the regeneration of tetrahydrofolate from 5-methyltetrahydro- folate. This conclusion was based on the observation that exogenous supplied homocysteine almost completely prevented the cytostatic activity of this nucleoside analogue.

HOMOCYSTEINE AND METHOTREXATE

Methotrexate is an antifolate drug which acts by inhibiting the enzyme dihydrofolate reductase; the enzyme responsible for regeneration of tetrahydrofolate from dihydrofolate (Jackson,1984). Methotrexate thereby induces cellular depletion of reduced folates, including 5-methyl - tetrahydrofolate, which serves as a methyl donor in the methionine synthase reaction (see figure 1). On the basis of these facts we investigated the effect of methotrexate on the disposition of endogenous homocysteine in cultured cells.

The mouse fibroblast cell line, C3H/10T1/2 Cl 8 (Cl 8) were less sensitive towards the cytotoxic effect of methotrexate than their malignant counterpart, MCA Cl 16 cells. Methotrexate increased the homocysteine secretion(up to 3 fold) from both cell types in a dose dependent manner, but about ten times higher concentrations were required for the enhancement of Hcy egress from the methotrexate resistant Cl 8 cells as compared with the sensitive Cl 16 cells. Thus, a correlation between Hcy egress and methotrexate cytotoxicity seems to exist. The effect of methotrexate on the release of Hcy into the extracellular medium was not associated with intracellular increase in AdoHcy. This suggests that intracellular Hcy is kept below the level required for reversal or inhibition of the AdoHcy hydrolase reaction. This is in accordance with the view that intracellular Hcy is tightly regulated.

The methotrexate dependent Hcy egress from Cl 16 cells is almost completely inhibited following "rescue" of these cells with 5-formyl - tetrahydrofolate, whereas a "rescue" therapy with thymidine plus

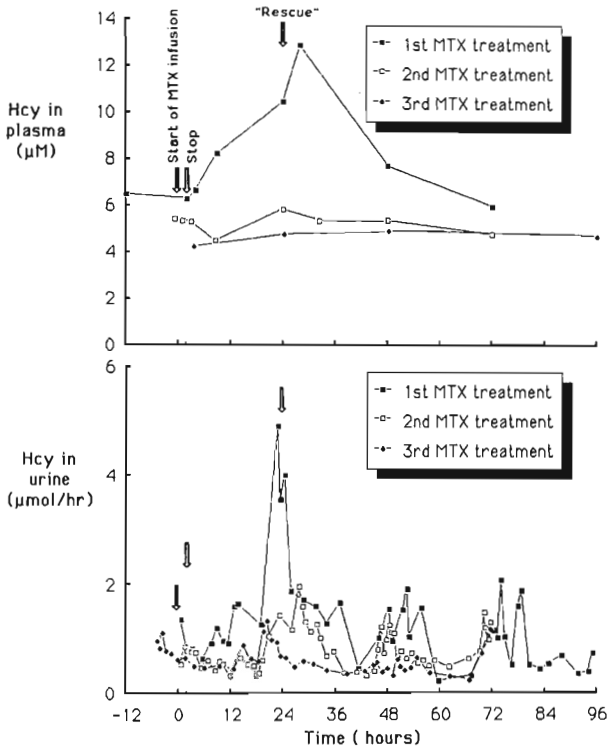

FIGURE 3: Total Hcy in plasma (upper panel) and urinary excretion of Hcy (lower panel) in a patient (K.A.L.) receiving high-dose treatment with MTX, followed by "rescue" with leucovorin (5-formyl-THF). The patient received infusion with high-dose MTX (220 mg/kg body weight) for 2 hours and "rescue" therapy with leucovorin (165 mg) at time 24 hours after initiation of MTX dosing. The treatment was repeated three times with 14 days of drug free intervals.

hypoxanthine (Jackson, 1984) had essentially no effect on the egress. Furthermore, cytotoxic agents other than methotrexate reduced rather than increased Hcy egress. These data show that stimulation of Hcy egress is not an effect of cytotoxic agents in general, but seems to be related to reduction of reduced folates relative to the metabolic demand.

Induction of homocysteine release from cells by methotrexate led us to investigate the homocysteine secretion in urine and Hcy content in plasma from patients receiving high-dose methotrexate treatment against

malignant disease. The data obtained were compared with normal values (Refsum et al., 1985) for these parameters in humans.

In some patients a drastic increase of Hcy in plasma was observed a few hours after methotrexate infusion, and this plasma peak was often associated with the secretion of copious amounts of Hcy in the urine. The Hcy response declined progressively after each treatment. Typical data (from patient K.A.L.) are shown in figure 3. The Hcy response varied markedly from one patient to another; some patients showed an increase in plasma content whereas in others an increase in urinary secretion predominated. The amount of methionine in plasma from these patients did not show similar variations.

It is conceivable that the release of Hcy from cells into the extracellular media like culture medium, plasma or urine, is a measure of depletion of reduced folates in target cells and thereby the cytotoxic effects of methotrexate. In this case, monitoring the Hcy response may provide useful information on metabolic effects of methotrexate therapy. This possibility is further investigated.

PERSPECTIVES

There are increasing numbers of reports showing that biological effects of nucleoside analogues interacting with AdoHcy hydrolase can be dissociated from inhibition of AdoMet dependent transmethylation reactions, induced by nucleosidylhomocysteine. Such data have been provided for 3-deazaadenosine and 3-deazaaristeromycin (Aksamit et al., 1982,1983; Garcia-Castro et al., 1983; Zimmerman et al., 1984).

Competitive inhibitors, irreversible inactivators and substrates of AdoHcy hydrolase may have similar effects on the metabolism of AdoHcy; all these compounds induce massive accumulation of AdoHcy. However, it is conceivable that adenosine analogues which serve as substrates, may condense with endogenous Hcy, and thereby cause effects on Hcy metabolism quite different from those induced by compounds which merely block the conversion of AdoHcy to Hcy.

Inhibition of the AdoHcy hydrolase reaction may reduce the rate of formation of Hcy and thereby the metabolic reactions dependent on Hcy. These include the conversion of 5-methyltetrahydrofolate to tetrahydrofolate (Cantoni et al., 1981) and synthesis of methionine, cystathionine and cysteine (Fig. 1). Furthermore, Hcy or its protein-mixed disulfide may play a role in metabolic regulation, and inhibition of Hcy formation may therefore have biological effects not hitherto recognized.

Adenosine analogues interacting with AdoHcy hydrolase and antifolate drugs seem to have a common intracellular target, in that both classes of compounds interfere with Hcy metabolism, albeit in the opposite

direction. This fact suggests interactions between adenosine analogues and antifolate drugs.

SUMMARY

1) Some adenosine analogues inhibit or inactivate the enzyme AdoHcy hydrolase. High affinity towards the intracellular enzyme and metabolic stability of the analogue are factors which seem critical for effectiveness *in vivo*.

2) Inhibition of AdoHcy hydrolase leads to accumulation of large amounts of intracellular AdoHcy, particularly in liver and kidney. Accumulation of AdoHcy is associated with release of copious amounts of AdoHcy into the extracellular medium.

3) Hcy is present in tissues as free Hcy and partly as Hcy associated with proteins (protein-bound Hcy). Hcy is exported into the medium from cultured cells or cells in suspension.

4) Inhibition of Hcy formation from AdoHcy by adenosine analogues is not associated with a reduction of the amount of Hcy in tissues or cells, but blocks the Hcy egress into the extracellular medium.

5) Hcy egress from cultured cells into the extracellular medium is greatly enhanced following exposure to the antifolate drug, methotrexate. The methotrexate dependent Hcy egress is probably related to cellular depletion of reduced folates, including 5-methyltetrahydrofolate required for the salvage of Hcy to methionine in most tissues.

6) The *in vitro* data (point 5) led us to investigate the amount of Hcy in extracellular media like plasma and urine from patients receiving high-dose methotrexate treatment against cancer. Both plasma content and urinary excretion of Hcy showed a transitory increase following methotrexate infusion. It is conceivable that the biological effects of methotrexate *in vivo* could be monitored from plasma and/or urinary Hcy.

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