Antiviral Research, 19 (1992) 247–265

AVR 00587

Rational approaches to the design of antiviral agents based on S-adenosyl-L-homocysteine hydrolase as a molecular target

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(Received 28 January 1992; accepted 3 June 1992)

S-adenosyl-L-homocysteine; S-adenosyl-L-homocysteine hydrolase; S-adenosylmethionine; Antiviral; Methylation

Introduction

S-Adenosyl-L-methionine (AdoMet)-dependent transmethylations play an important role in regulating various biochemical and physiological processes (Borchardt, 1980; Borchardt et al., 1986). A specific biochemical process of interest to our laboratory is the formation of the 'capped methylated structure' at the 5'-terminus of viral mRNA (Keller and Borchardt, 1986). Most viral mRNAs have been found to contain this novel 5'-terminal capped structure, which is essential for viral mRNA translation and thus viral replication in eukaryotic cells (Banerjee, 1980). All capped methylated structures consist of a *N*-7-methyl guanosine residue linked at the 5'-hydroxyl group to the 5'-end of the mRNA strand by a triphosphate linkage. Most capped methylated structures also contain a methyl group on the 2'-hydroxyl group of the penultimate nucleotide (Fig. 1a). Methylations at the 5'-terminus of mRNA are catalyzed by AdoMet-dependent guanosine 7-*N*-methyltransferase and nucleoside 2'-methyltransferase, which are both cellular and viral encoded enzymes (Fig. 1b).

S-Adenosylhomocysteine (AdoHcy), the product of these AdoMet-dependent methyltransferases, has been shown to be a potent competitive product

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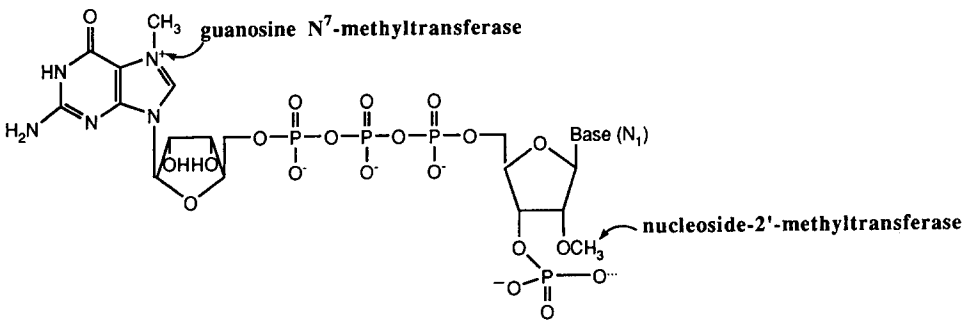


Figure 1a. Structure of mRNA 5'-terminal Cap.

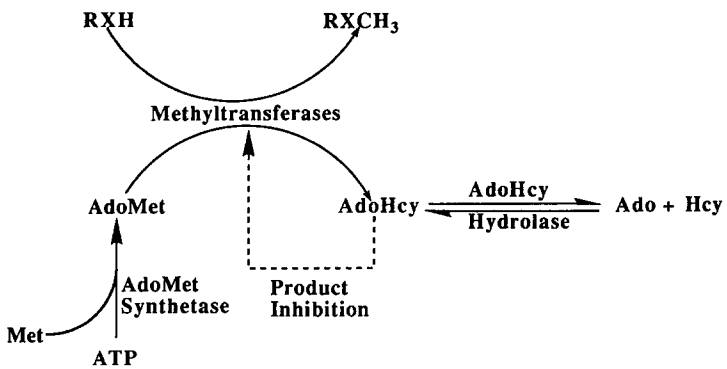


Figure 1b. General Scheme for AdoMet-Dependent Methyltransferases

inhibitor of all methyltransferases (Cantoni and Chiang, 1980; Chiang and Cantoni, 1979), including the enzymes involved in methylation of the capped methylated structure at the 5'-terminus of mRNA (Keller and Borchardt, 1986, 1988; Ransohoff et al., 1987). To sustain AdoMet-dependent transmethylation reactions, AdoHcy is metabolized by cellular AdoHcy hydrolase (EC 3.3.1.1) to adenosine (Ado) and homocysteine (Hcy) (Fig. 1b) (Borchardt, 1980; Wolfe and Borchardt, 1991). Inhibition of AdoHcy hydrolase results in cellular accumulation of AdoHcy, which slows transmethylation reactions, including those involved in viral mRNA methylations (Keller and Borchardt, 1986, 1988; Ransohoff et al., 1987). Because of the critical role of methylation reactions in viral replication and the proven antiviral effects of AdoHcy hydrolase inhibitors, this enzyme has become a very attractive target for the design of antiviral agents (De Clercq, 1987; Keller and Borchardt, 1988; Wolfe and Borchardt, 1991).

Considerable evidence now exists to support the hypothesis that certain Ado analogs (e.g., 3-deazaadenosine (c^3-Ado)) and carbocyclic Ado analogs (e.g., neplanocin A (NpcA), 3-deazaNpcA (c^3-NpcA), aristeromycin (Ari), 3-deazaAri) produce their antiviral activity by inhibition of cellular AdoHcy

hydrolase. For example, Cools and De Clercq (1989) have shown a strong correlation between the inhibitory effects of Ado analogs toward AdoHcy hydrolase and their antiviral activity. In addition, the antiviral activities of AdoHcy hydrolase inhibitors have been correlated with their ability to elevate the cellular levels of AdoHcy (Hasobe et al., 1989a; Cools and De Clercq, 1990). These analogs have been shown to inhibit the replication of a variety of viruses including DNA viruses, (−) RNA viruses and double-stranded RNA viruses (De Clercq, 1985, 1987; De Clercq et al., 1989).

The purpose of this manuscript is to review ways in which enzymology, molecular biology and computational chemistry are being employed to rationally design more potent inhibitors of AdoHcy hydrolase that are less cytotoxic and thus potentially more useful as antiviral agents.

Structure of AdoHcy hydrolase and its mechanism of catalysis

AdoHcy hydrolase catalyzes the reversible hydrolysis of AdoHcy to Ado and Hcy. In vitro the reaction favors the synthetic direction; however, in vivo the hydrolytic reaction is favored because Ado and Hcy are rapidly metabolized (Cortese et al., 1974; Crooks et al., 1979; De la Haba et al., 1959). The mammalian AdoHcy hydrolase exists as a tetramer (Richards et al., 1978; Fujioka and Takata, 1981; Doskeland and Ueland, 1982) with approximately one molecule of tightly bound NAD^+ per subunit. The complete amino acid sequences of rat liver and human placental AdoHcy hydrolases have been deduced from the nucleotide sequences of cloned cDNAs (Ogawa et al., 1987; Coulter-Karis and Hershfield, 1989). It was found that the amino acid sequences for rat liver and human placenta AdoHcy hydrolases are 97% homologous (Coulter-Karis and Hershfield, 1989).

The determination of the amino acid sequence of the hydrolase from these clones paved the way for the development of computer-generated molecular graphics of the active sites of the rat liver and human placental enzymes. Lactate dehydrogenase (LDH), malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and liver alcohol dehydrogenase (all NAD^+-containing enzymes) have similar three-dimensional structures in their dinucleotide-binding domains, even though their primary sequences and the locations of these binding domains within the primary sequences are quite different (Branden and Eklund, 1980; Birktoft and Banaszak, 1984). Since the tertiary structure of AdoHcy hydrolase is presently unknown and crystals of necessary purity for X-ray diffraction have proven elusive, a computer model of the enzyme was developed in our laboratory by computational mutation of LDH, a functionally similar (i.e., NAD^+-binding) protein of known structure (Yeh et al., 1991). Fig. 2a illustrates the binding of the substrate, Ado, and the cofactor, NAD^+, at the proposed active site of AdoHcy hydrolase. Similarly, Fig. 2b illustrates the binding of an inhibitor, NpcA (see Fig. 4 for structure), and the cofactor NAD^+ at the enzyme active site. This computer model of the

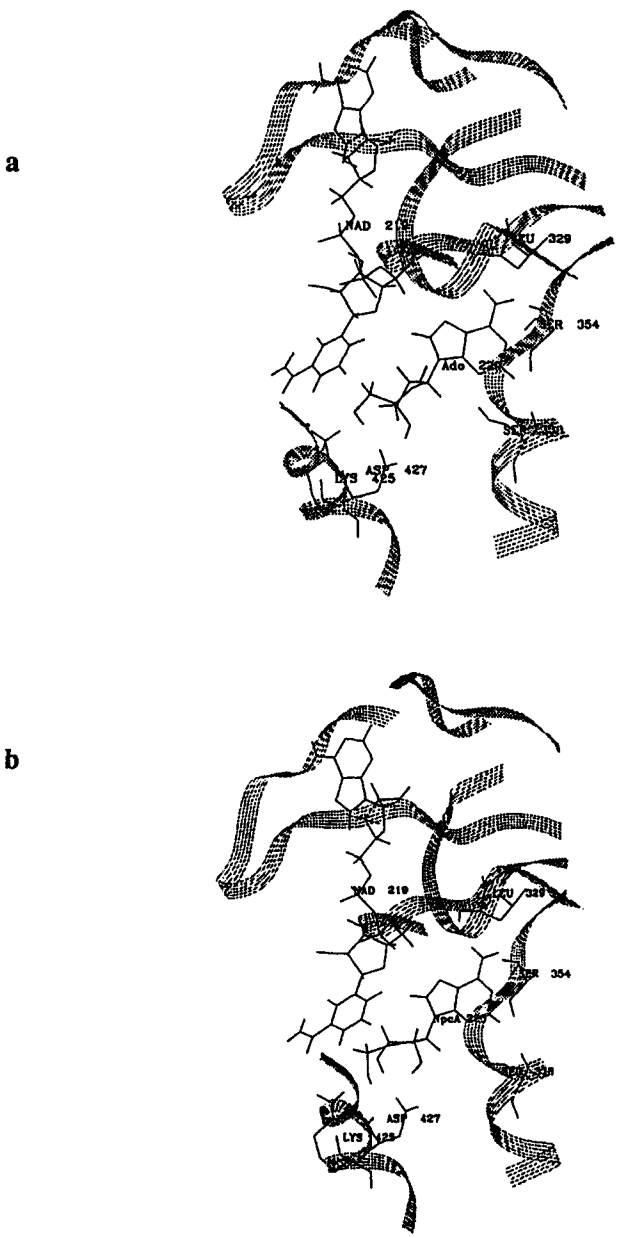


Figure 2. (a) Computer graphics models of a substrate (Ado) docked at the active site of AdoHyy hydrolase. (b) Computer graphics models of an inhibitor (NpcA) docked at the active site of AdoHcy hydrolase.

active site has proven useful in predicting the inhibitory activity of a series of carbocyclic nucleosides (Yeh et al., 1991) and in aiding in the design of more potent inhibitors of AdoHcy hydrolase (unpublished data).

The mechanism of the enzymatic reaction as proposed by Palmer and Abeles (1976, 1979) involves the reversible oxidation-reduction of the nucleoside

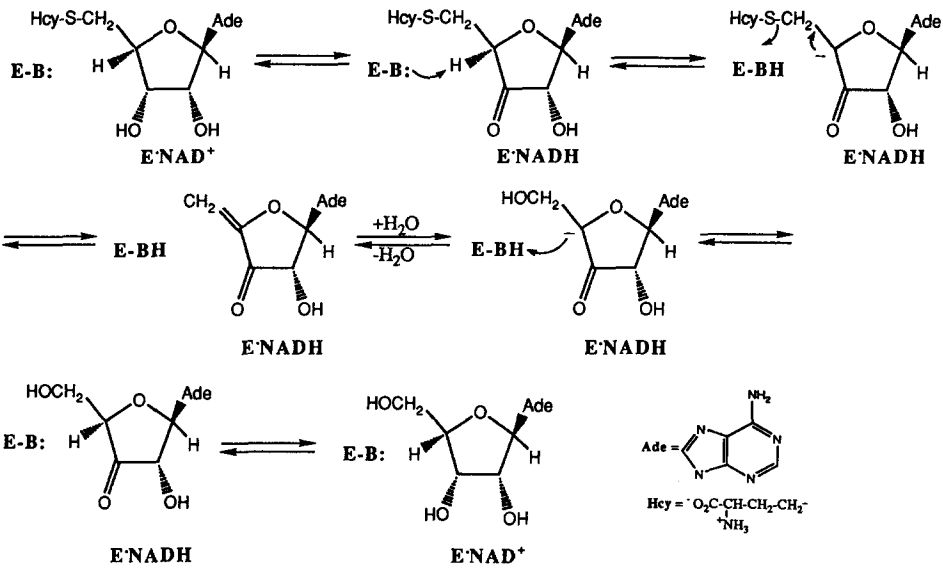


Figure 3. Catalytic mechanism for AdoHcy hydrolase as proposed by Palmer and Abeles (1979).

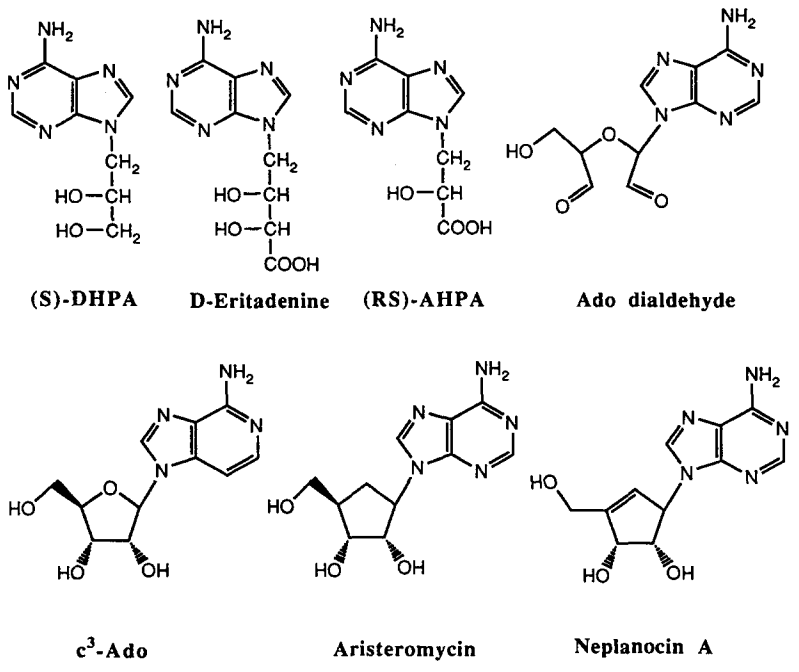


Figure 4. First-generation of AdoHcy hydrolase inhibitors.

substrate by the enzyme-bound cofactor, NAD^+ (Fig. 3). Oxidation of the 3′-hydroxyl group of AdoHcy by NAD^+ produces 3′-keto-AdoHcy. The 3′-keto group increases the acidity of the C-4′ proton, allowing for abstraction of this proton by a base in the active site of the enzyme. Subsequently, elimination of the C-5′ substituent (e.g., homocysteinyl group) results in the formation of the intermediate 3′-keto-4′,5′-dehydro-Ado. Addition of water to the 5′-position of this intermediate gives 3′-keto-Ado. This ketone is then reduced by the enzyme-bound NADH, resulting in Ado and regenerating the NAD^+ form of the enzyme, which is now ready for another round of catalysis. The enzyme can also catalyze the reverse reaction (synthesis of AdoHcy), using Ado and Hcy as substrates.

Inhibitors and their mechanism of inactivation of AdoHcy hydrolase

AdoHcy hydrolase can be inhibited by a variety of acyclic and carbocyclic adenosine analogs (see Keller and Borchardt, 1988; Wolfe and Borchardt, 1991 for comprehensive reviews). The first-generation AdoHcy hydrolase inhibitors (Fig. 4) included Ado analogs with acyclic 'sugar' moieties such as 9(S)-(2,3-dihydroxypropyl)adenine ((S)-DHPA) (De Clercq et al., 1978; De Clercq and Holy, 1979; Votruba and Holy, 1980; Merta et al., 1983), D-eritadenine (Holy et al., 1982; Votruba and Holy, 1982; Merta et al., 1983; De Clercq et al., 1984), (R,S)-3-adenine-9-yl-2-hydroxypropanoic acid ((R,S)-AHPA) (De Clercq and Holy, 1985; Holy et al., 1985) and adenosine dialdehyde (Grant and Lerner, 1979; Borchardt et al., 1982; Bartel and Borchardt, 1984; Patel and Borchardt, 1985). Ado analogs with ribose or carbocyclic ribose moieties were also effective inhibitors; e.g., c^3-Ado (Bader et al., 1978; Chiang et al., 1978; Bodner et al., 1981; Guranowski et al., 1981; Kim et al., 1983), and the carbocyclic nucleosides Ari (Hill et al., 1971; Guranowski et al., 1981) and NpcA (Borchardt et al., 1984). Some of these compounds are quite potent, with K_i values for AdoHcy hydrolase below 10 nM; unfortunately, the cytotoxicity of several of these compounds (e.g., NpcA) precluded their clinical use as antiviral agents (De Clercq, 1985, 1987).

In the mid-1980s, several laboratories undertook studies of the mechanisms by which the compounds shown in Fig. 4 inhibit AdoHcy hydrolase. It was felt that such information could be helpful in the design of the second-generation of AdoHcy hydrolase inhibitors, which would be more potent and more specific. Of particular interest in our laboratory was NpcA, a naturally occurring anticancer agent (Yaginuma et al., 1980, 1981; Hayashi et al., 1981). Our laboratory demonstrated that NpcA is a potent inhibitor of the bovine enzyme and an inhibitor of vaccinia virus replication in mouse L929 cells (Borchardt et al., 1984). Subsequently, NpcA was shown to be a broad spectrum antiviral agent (De Clercq, 1985, 1987). Its antiviral activity has been correlated with its ability to inhibit AdoHcy hydrolase (Cools and De Clercq, 1989) and its ability to elevate cellular levels of AdoHcy (Hasobe et al., 1989a; Cools and De Clercq,

1990).

NpcA was of particular interest as an AdoHcy hydrolase inhibitor because it was potent (K_i = 3 nM) and produced time-dependent inactivation of the enzyme (Borchardt et al., 1984; Matuszewska and Borchardt, 1987a,b). The mechanism of NpcA-induced inactivation of AdoHcy has been shown to involve reduction of the enzyme-bound NAD^+ to NADH with simultaneous oxidation of NpcA to 3′-keto NpcA (Fig. 5). The fact that the inactivation of the enzyme by NpcA is almost completely reversed by subsequent incubation of the enzyme with NAD^+ (Matuszewska and Borchardt, 1987a,b) suggested that the mechanism could be due to the depletion of the enzyme-bound cofactor NAD^+. This mechanism is thus referred to as a 'cofactor depletion mechanism,' and inhibitors which inactivate the enzyme in this way have been designated as type I mechanism-based inhibitors (Fig. 5) (Wolfe and Borchardt, 1991).

Further investigation in our laboratory provided more evidence to support this cofactor depletion mechanism for the inhibition of AdoHcy hydrolase by NpcA (Paisley et al., 1989a). The intermediate 3′-keto-NpcA was isolated and characterized. Examination of the physicochemical properties of 3′-keto-NpcA (Paisley et al., 1989a) and a synthetic analog (3′-keto-DHCeA) (Paisley et al., 1989b) showed that both 3′-keto carbocyclic nucleosides are stable in acidic conditions, but rapidly decompose in neutral and alkaline media, releasing

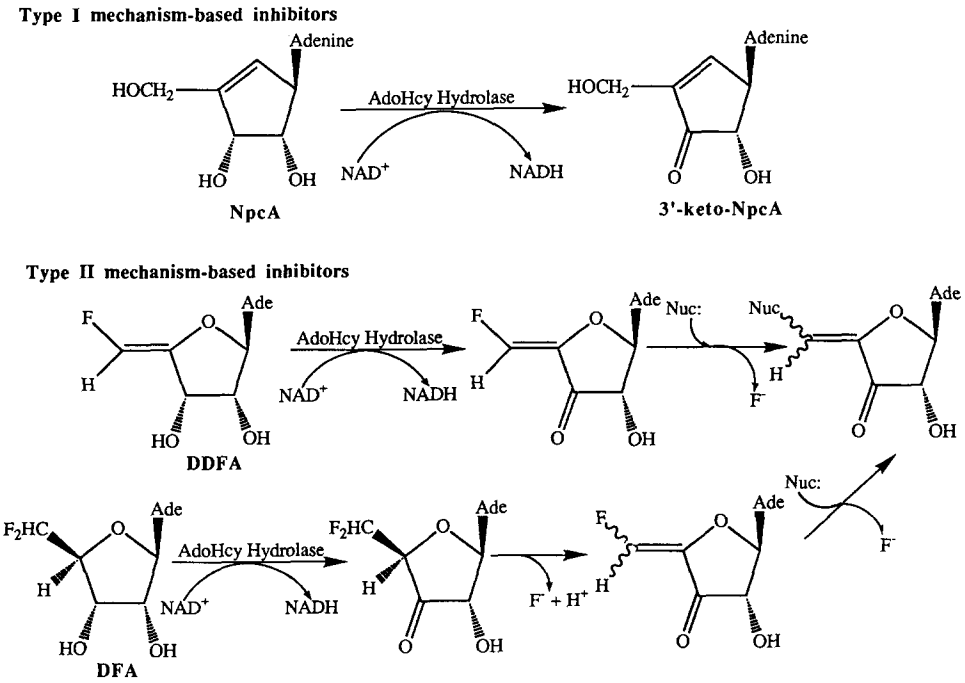
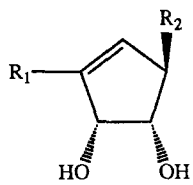
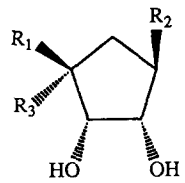


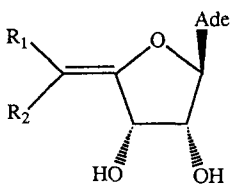
Figure 5. Mechanism for AdoHcy hydrolase inhibition.



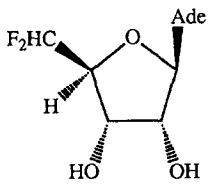
DHCeA R_1: H, R_2: Ade
c³-DHCeA R_1: H, R_2: c^3Ade



DHCaA R_1=R_3 : H, R_2: Ade
c³-DHCaA R_1=R_3 : H, R_2: c^3Ade
β-4'-Me-DHCaA R_1: CH_3, R_3: H, R_2: Ade
β-4'-Vinyl-DHCaA R_1: CH_2=CH, R_3: H, R_2: Ade



Z-DDFA R_1: F, R_2: H
E-DDFA R_1: H, R_2: F



DFA

Figure 6. Second-generation AdoHcy hydrolase inhibitors.

adenine. These observations are important because Wolfson et al. (1986) had suggested earlier that release of adenine and formation of a strong electrophile at the active site of the enzyme was integral to the enzyme inactivation process. Apparently, the release of adenine reported by Wolfson et al. (1986) was caused by the instability of the 3′-keto nucleoside rather than such release being an indication of an important step in the mechanism of enzyme inactivation.

Based on the results of these mechanistic studies with NpcA, as well as cellular metabolism studies with this carbocyclic nucleoside (see Discussion below), a series of second-generation AdoHcy hydrolase inhibitors (Fig. 6; 9-(*trans*-2′-,*rans*-3′-dihydroxycyclopent-4′-enyl)adenine (DHCeA); 3-deaza-DHCeA (c^3-DHCeA); 9-(*trans*-2′-*trans*-3′-dihydroxycyclopentanyl)adenine (DHCaA); 3-deaza-DHCaA (c^3-DHCaA); β-4′-methyl-DHCaA; β-4′-vinyl-DHCaA) have been synthesized and shown to inactivate the enzyme by this type I mechanism (Fig. 5) (Borcherding et al., 1987; Narayanan et al., 1988; Paisley et al., 1989b; Wolfe et al., 1992; Yuan et al., unpublished data).

Recently, another type of AdoHcy hydrolase inhibitor, designated as type II mechanism-based (Fig. 6; (Z)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (Z-DDFA); (E)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (E-DDFA); 5′-deoxy-5′-difluoroadenosine (DFA)) has been developed by McCarthy and coworkers (McCarthy et al., 1989; Mehdi et al., 1990). As in the case of type I mechanism-

based inhibitors, type II mechanism-based inhibitors are first oxidized to the 3′-keto intermediate and NAD^+ is reduced to NADH. However, the presence of a halide substituent (e.g., fluoride) at the 5′-position of the 3′-keto intermediate apparently leads to attack by a nucleophile, resulting in release of fluoride ion (Fig. 5). If the nucleophile is an enzyme nucleophile (e.g., lysine amino group), a covalent bond could be formed between the inhibitor and the enzyme. However, the nucleophile could simply be a water molecule. If so, the mechanism for such inhibition is basically the same as for type I mechanism-based inhibitors (i.e., tight binding of the oxidized inhibitor to the NADH form of the enzyme). Further studies are needed to clarify the mechanism of inactivation of AdoHcy hydrolase by the fluoronucleosides.

AdoHcy hydrolase inhibitors: antiviral effects vs. cytotoxicity

As mentioned above, one of the major limitations of the first generation of AdoHcy hydrolase inhibitors (e.g., NpcA) was their cytotoxicity (De Clercq, 1985, 1987). Since AdoHcy hydrolase is a cellular enzyme, it was extremely important to determine whether this cytotoxicity was due to inhibition of AdoHcy hydrolase or to other cellular interactions.

Clues concerning the nature of this toxicity were provided by cellular metabolism studies on NpcA (Fig. 7, and Discussion below) and Ari (see Wolfe and Borchardt (1991) for a comprehensive review). In early studies, our laboratory showed that a concentration as low as 0.1 μM NpcA inactivates 90% of the hydrolase in murine L929 cells, resulting in a marked increase in

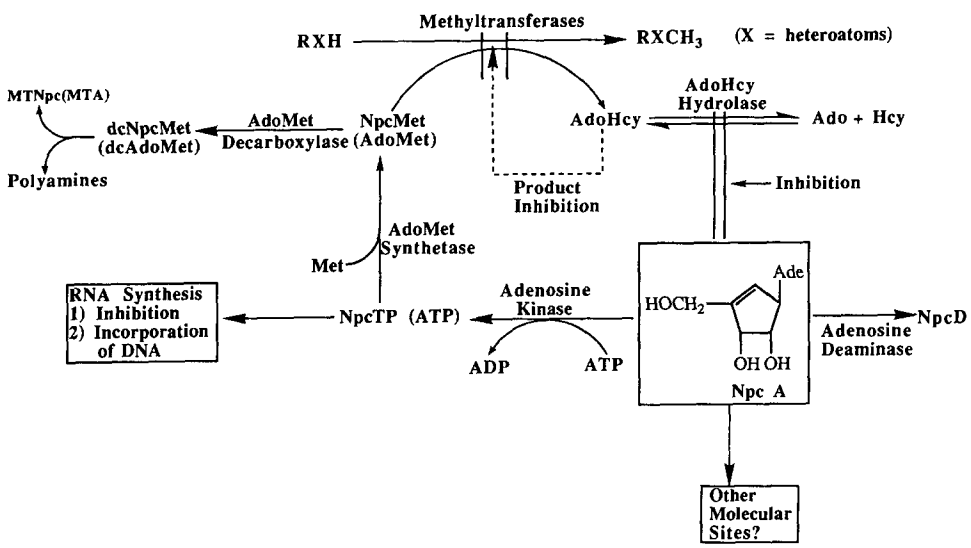


Figure 7. Metabolic Pathways of NpcA (Normal Ado Metabolites in Parentheses).

intracellular ratio of AdoHcy/AdoMet (Borchardt et al., 1984). While not a substrate for the hydrolase, NpcA is converted by Ado deaminase to the biologically inactive neplanocin D, the cyclopentenyl counterpart of inosine (Tsujino et al., 1980; Keller and Borchardt, 1986). However, this metabolic route does not seem to be important for the biological activity of NpcA, since coadministration of deoxycoformycin or EHNA (both Ado deaminase inhibitors) did not potentiate the effect of NpcA in several different cell lines (Keller and Borchardt, 1986; Saunders et al., 1985; Glazer and Knode, 1984).

NpcA is also converted to its corresponding 5′-triphosphate (NpcTP), presumably via Ado kinase, and, subsequently, adenylate kinase and nucleoside diphosphokinase (Glazer and Knode, 1984; Saunders et al., 1985; Whaun et al., 1986). The AdoMet counterpart of NpcA (NpcMet) is formed as well (Glazer and Knode, 1984; Keller and Borchardt, 1984; Linevsky et al., 1985; Whaun et al., 1986; Ramakrishnan and Borchardt, 1987), presumably via NpcTP and AdoMet synthetase. Deleterious effects, which arise due to the formation of these metabolites, are apparently a function of the cell line employed. For instance, cytocidal activity in HT-29 human colon carcinoma cells seems to result from formation of NpcMet (Glazer et al., 1986). In murine leukemia cells, a NpcA-resistant cell line possessed decreased Ado kinase activity, suggesting that 5′-phosphorylation leads to the antitumor activity observed in the normal cell line (Inaba et al., 1986). On the other hand, Chinese hamster ovary (CHO) cells are known to metabolize NpcA to NpcTP, but an Ado kinase mutant ($AdoK^-$) cell line (which produced little, if any, NpcTP) was only slightly more resistant to NpcA treatment (Saunders et al., 1985). Not much is known about the mechanism of toxicity of NpcTP, except that it is converted to NpcMet and is minimally incorporated into RNA (Glazer and Knode, 1984). Given the ubiquity of the involvement of ATP in biological processes, however, it would not be surprising to find NpcTP interfering with some fundamental cellular process.

With respect to the toxicity of NpcMet per se, studies by Glazer and Knode (1984) and Glazer et al. (1986) suggest that it may inhibit cellular RNA methylation (HT-29 cells) and that NpcA may exert its toxic effect through this metabolite. To date, the effect of NpcMet on mRNA methyltransferase has not been examined. Work in our laboratory revealed that NpcMet is only a weak inhibitor of cellular lipid methylation and protein carboxymethylation and is not a substrate for either enzyme involved in these transformations (Keller and Borchardt, 1984, 1986). Also, NpcMet is neither a substrate nor an inhibitor of AdoMet decarboxylase (Keller and Borchardt, 1984, 1986); however, it did serve as a substrate for catechol-*O*-methyltransferase (Keller and Borchardt, 1984). The assumed formation of NpcHcy in this last case raises the question of whether this metabolite plays a role in the observed antiviral activity and/or cytotoxic effects of NpcA in some cell lines by inhibition of viral or cellular methyltransferases.

These studies with NpcA and similar studies with Ari (see Wolfe and Borchardt (1991) for comprehensive review) raised questions as to whether the

antiviral activity of NpcA (and Ari) was the result of increased AdoHcy levels and/or the result of the formation of these metabolites. To answer this question, several laboratories undertook the preparation of NpcA and Ari analogs that would be devoid of substrate activity toward adenosine kinase and adenosine deaminase but retain inhibitory activity toward AdoHcy hydrolase. These studies led to the design and synthesis of second-generation inhibitors of AdoHcy hydrolase which are potent antiviral agents with reduced cytotoxicities.

Rational approaches to the design of more specific inhibitors of AdoHcy hydrolase

One approach to the design of more specific inhibitors of AdoHcy hydrolase was to replace the adenine moiety of NpcA and Ari with 3-deazaadenine, resulting in c^3-NpcA (Glazer et al., 1986; Tseng et al., 1989) and c^3-Ari (Montgomery et al., 1982), respectively. These compounds were shown to lack substrate activity toward Ado kinase and Ado deaminase, but still retain potent inhibitory activity toward AdoHcy hydrolase. In addition, these compounds were shown to have potent antiviral activity with reduced cytotoxicity compared to that of the parent carbocyclic nucleosides (De Clercq et al., 1989).

Another approach to enhancing the specificity of AdoHcy hydrolase inhibitors was the actual removal of the hydroxymethyl substituent, which would preclude 5′-phosphorylation by Ado kinase and deamination by Ado deaminase (Bloch et al., 1967). The resultant analog of NpcA (DHCeA, Fig. 6) and its 3-deaza counterpart (c^3-DHCeA, Fig. 6), synthesized in our laboratory (Borcherding et al., 1987), did not in fact serve as a substrate for these two enzymes; however, they did retain potent inhibitory activity against AdoHcy hydrolase (Hasobe et al., 1987, 1988; Narayanan et al., 1988). Their saturated counterparts (DHCaA, c^3-DHCaA), as well as β-4′-methyl DHCaA, β-4′-vinyl DHCaA, also proved to be potent and selective hydrolase inhibitors (Wolfe et al., 1992). These 4′-modified analogs of NpcA (DHCeA, c^3-DHCeA) and Ari (DHCaA, c^3-DHCaA, β-4′-methyl DHCaA, β-4′-vinyl DHCaA, Fig. 6) all apparently inactivate AdoHcy hydrolase by a type I mechanism, involving reduction of NAD^+ to NADH (Wolfe and Borchardt, 1992; Yuan et al., unpublished data).

These second-generation AdoHcy hydrolase inhibitors also retain antiviral activity while their cytotoxicity is considerably lower than that of the parent compounds (Table 1) (Hasobe et al., 1987; Hasobe et al., unpublished data; De Clercq et al., 1989). For example, NpcA is a more potent inhibitor of Vaccinia virus replication (EC_{50}) in murine L929 fibroblast cells than DHCeA by a factor of 3.5; however, DHCeA is 34-times less cytotoxic (measured as the IC_{50} value, the concentration of drug which causes 50% inhibition of cellular replication) (Hasobe et al., 1987). DHCeA is, therefore, a better antiviral agent than NpcA by a factor of 10. By this criterion, the Ari analogs DHCaA and c^3-

TABLE 1

Antiviral activity and cytotoxicity of selective S-adenosyl-L-homocysteine hydrolase inhibitors

Compounds	Antiviral activity and index		Cytotoxicity and index		Antiviral effectiveness
	EC_{50} (μM)	EC_{50} (analogue) /EC_{50} (parent)	IC_{50} (μM)	IC_{50} (analogue) /IC_{50} (parent)	(IC_{50}/EC_{50})
DHCeA	0.28	3.5	17	34	61
c^3-DHCeA	0.95	11.9	56	112	59
NpcA	0.08	1	0.5	1	6
DHCaA	0.17	0.03	73	17	429
c^3-DHCaA	0.13	0.02	27.4	6	211
Ari	6.62	1	4.3	1	0.64
E-DDFA	2.96	–	>100	–	>34
Z-DDFA	0.05	–	15.6	–	312

DHCaA were also much better antiviral agents than Ari by factors of 670 and 330, respectively (Hasobe et al., unpublished data). Apparently, elimination of Ado kinase and Ado deaminase substrate activity led to the reduced toxicity of these analogs.

These second-generation AdoHcy hydrolase inhibitors have added to the body of evidence indicating that inhibition of this enzyme results in the inhibition of viral replication. The design of selective inhibitors of AdoHcy hydrolase has also led to Ado analogs with potent broad-spectrum antiviral activity but reduced cytotoxicity in comparison to less selective analogs.

Further studies

New techniques in molecular biology and computational chemistry are being utilized in order to design more potent AdoHcy hydrolase inhibitors which retain the selectivity of the second-generation inhibitors. The cloning of AdoHcy hydrolase from rat liver (Ogawa et al., 1987) and human placenta (Coulter-Karis and Hershfield, 1989) has allowed the determination of their respective amino acid sequences. The homology between the hydrolase primary sequences and the primary sequences of NAD^+-dependent dehydrogenases (e.g., LDH) has permitted the generation of computer models of the active sites of rat and human AdoHcy hydrolases by computational mutation of LDH (Yeh and Borchardt, 1991). This model has proven useful in designing structures that have enhanced affinity for the active site of this enzyme (Yeh et al., 1991). More potent (better binding) S-adenosyl-L-homocysteine hydrolase inhibitors might produce comparable antiviral effects at lower concentrations. At these lower concentrations, toxicity resulting from molecular interactions other than hydrolase inhibition might be attenuated. The overall effect would be to increase the antiviral selectivity of AdoHcy hydrolase inhibitors.

Molecular biological techniques may also help solve another difficult problem in AdoHcy hydrolase inhibitor design. In cellular systems, even the most potent in vitro AdoHcy hydrolase inhibitors do not inhibit 10–15% of the enzyme (Bartel et al., 1984; Schanche et al., 1984; Hasobe et al., 1989b). This could be explained by the existence of separate 'pools' of the hydrolase, some of which may be inaccessible to Ado analogs; or by the presence of multiple isoforms of the enzyme, one inhibitor-sensitive and the other inhibitor-insensitive. In fact, two forms of AdoHcy hydrolase have been separated by chromatographic means (Lee et al., 1990). These isoenzymes may explain why the antiviral effects of a given hydrolase inhibitor can vary up to 200-fold, depending upon the type of cultured host cells used for viral replication studies (De Clercq, 1985).

The interest in the different isoforms of the hydrolase stems not only from a desire to design more effective antiviral agents but also from a curiosity about the roles which these isoforms may play in the regulation of AdoHcy metabolism. Are the isomers the result of different genes, alternate mRNA splicing or post-translational modification of a single gene product? How is the expression of the different isomers regulated? What are the physiological roles of these different isoforms?

Another important question which needs to be addressed deals with the biosynthetic source of AdoHcy in cells treated with an inhibitor of AdoHcy hydrolase. The answer appears to be not only AdoMet (via methyltransferases) but also Ado and Hcy (via AdoHcy hydrolase). Double-labeling experiments using [^{3}H]Ado and [^{35}S]methionine showed that the ratio [^{3}H]/[^{35}S] was higher in AdoHcy than AdoMet in cells inhibited with a selective hydrolase inhibitor (e.g., DHCeA), suggesting that the inhibitor-insensitive form of AdoHcy hydrolase contributes to the biosynthesis of AdoHcy. This knowledge is not of academic interest alone. Hcy potentiates the antiviral effects of hydrolase inhibitors, and the above information suggests that this synergistic effect may be due to the conversion of this amino acid to AdoHcy via the hydrolase. In fact, with a hydrolase inhibitor and Hcy, increases in cellular AdoHcy/AdoMet to levels unattainable with drug alone have been observed (Hasobe et al., 1989b; Cools et al., 1990). Unfortunately, the additional Hcy also potentiates the cytotoxic effects of these compounds; however, the antiviral potentiation is greater. The overall result is an increase in the therapeutic effectiveness (IC_{50}/EC_{50}) of the Ado analog (Hasobe et al., 1989b; Cools et al., 1990).

Finally, one of the main objections to the use of AdoHcy hydrolase as a target for the development of antiviral agents is that there may be intrinsic toxicity associated with inhibiting such a key regulator of cellular function. This is certainly a valid concern, but until recently it was difficult to address this question because of the lack of specific hydrolase inhibitors. With the advent of compounds such as DHCeA, DHCaA and c^3-NpcA, the intrinsic toxicity of hydrolase inhibition has been approximated. The use of selective AdoHcy hydrolase inhibitors led to the discovery of a relationship between the ratio AdoHcy/AdoMet and cellular toxicity (Hasobe et al., 1989a). This ratio

was determined at the IC_{50} values of a variety of hydrolase inhibitors, and a consistent ratio of around 1.3 was observed in murine L929 cells. This means that AdoHcy hydrolase can be inhibited to levels which the cell cannot handle (that is, to the point where AdoHcy/AdoMet levels reach 1.3 or above), and the observed cytotoxicity might be due to inhibition of crucial cellular methyltransferases. Meanwhile, the determination of this ratio at the EC_{50} values revealed that the ratio is consistently 0.15–0.20 (Hasobe et al., 1989a). Although, so far, there has been no evidence for any virus-specific AdoHcy hydrolase, that it is apparently possible to achieve antiviral effectiveness by inhibiting AdoHcy hydrolase while keeping the cytotoxicity to a minimum. It is important to mention here that different cell lines or cells in different stages of differentiation may have different levels of tolerance to inhibitors of AdoHcy hydrolase. The existence of alternative pathways for AdoHcy metabolism or mechanisms for efflux of this nucleoside and the cellular levels of AdoMet may all contribute to the sensitivity of a cell line to the toxic effects of an AdoHcy hydrolase inhibitor.

It is also important to point out that even with the more potent, more specific second-generation AdoHcy hydrolase inhibitors, the difference between the EC_{50} and IC_{50} values for hydrolase inhibitors has not reached three orders of magnitude (Hasobe et al., unpublished data). If the cytotoxicity of these compounds is due solely to hydrolase inhibition (and this may not be so), then the relative difference between the EC_{50} and IC_{50} cannot be improved by designing and synthesizing a more potent hydrolase inhibitor because the IC_{50} value would be expected to drop along with the EC_{50} value with no net increase in therapeutic effectiveness. If this is the case, then improved therapeutic effectiveness would only be possible through combination with drugs which possess a different mechanism of action.

Combination drug studies have been performed with hydrolase inhibitors. Hcy is one such compound, which was mentioned earlier (Hasobe et al., 1989b; Cools and De Clercq, 1990). Two other compounds which have been examined are L-2-amino-4-methoxy-*cis*-but-3-enoic acid (L-*cis*-AMB) and ribavirin. L-*cis*-AMB is an inhibitor of AdoMet synthetase (Sufrin et al., 1982). Inhibition of this enzyme would be expected to decrease AdoMet levels in the cell, synergistically increasing AdoHcy/AdoMet and thereby producing a synergistic antiviral effect. This combination did, in fact, produce such a synergistic effect (Liang et al., unpublished data). Ribavirin was selected because it is a broad-spectrum antiviral agent whose effects are thought to be mediated, in part, by inhibition of GTP-dependent capping of the 5′-end of viral mRNA. When the antiviral activity (Vaccinia virus; murine L-929 cells) of c^3-DHCeA was determined in the presence of ribavirin, the antiviral effectiveness (IC_{50}/EC_{50}) of c^3-DHCeA increased 10-fold (Ishii et al., unpublished data). These results suggest that even if hydrolase inhibitors cannot be used clinically by themselves, they may be of practical use in combination with other agents.

Conclusion

The development of third-generation AdoHcy hydrolase inhibitors will rely heavily on new techniques in molecular biology and computational chemistry. These techniques will aid medicinal chemists in designing very potent inhibitors which retain selectivity for AdoHcy hydrolase. The main stumbling block to clinically useful AdoHcy hydrolase inhibitors may be their cellular toxicity. This problem may be overcome in part by combination regimens. For example, the discovery that Hcy and the antimetabolites L-*cis*-AMB and ribavirin potentiate the antiviral activity and therapeutic effectiveness of AdoHcy hydrolase inhibitors may lead to advances in the practical use of these Ado analogs in clinical situations. Even if these effects do not come to fruition in the form of marketable drugs, the development of potent and selective AdoHcy hydrolase inhibitors will provide the molecular tools needed by those who wish to determine the physiological function of this ubiquitous enzyme.

Acknowledgements

The authors' research on AdoHcy hydrolase has been supported by grants from the National Institute of General Medical Science (GM 29332) and Glaxo Inc.

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