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Perspective

S-Adenosyl-L-homocysteine Hydrolase as a Target for Antiviral Chemotherapy

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I. Introduction

It was only a few decades ago that the search for antiviral agents was widely thought to be an exercise in futility. Most virologists believed that viral replication was too intimately involved with cellular processes for "magic bullets" specific for these pathogens to be much more than fantasy. This dogma has since passed on with the advent of clinically useful antiviral agents, but the success of these agents has borne yet another firmly held tenent: antiviral agents should selectively interact with viral macromolecules (i.e., these agents should not interfere with normal cellular metabolic processes).[1] This assumption certainly appears practical as well as logical since the cause of a given toxic side effect can often be traced to the disturbance of some fundamental cell function.

A common mechanistic motif of nucleoside antiviral agents currently in clinical use is their metabolic conversion to the corresponding triphosphates and subsequent selective inhibition of viral nucleic acid polymerases. For example, zidovudine (3'-azidothymidine, AZT, Retrovir) is converted by cellular kinases to its 5'-triphosphate, and at therapeutic doses this metabolite selectively inactivates the reverse transcriptase of human immunodeficiency virus (HIV) while leaving cellular polymerases relatively unaffected.[2,3] Likewise, the triphosphate metabolite of acyclovir (9-[(2-hydroxyethoxy)methyl]guanine, Zovirax) selectively inhibits the DNA polymerase of herpes simplex virus type I (HSV I). In fact, this guanosine analogue is doubly selective for virus-encoded enzymes since the drug requires herpesvirus thymidine kinase for efficient conversion to the active metabolite.[4-7] Hence, the triphosphate of acyclovir only appreciably accumulates in herpes-infected cells.

Another example of this theme is found in ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Virazole). A broad-spectrum antiviral agent currently used in the treatment of respiratory syncyntial virus infection,[8] this nucleoside analogue must also be activated by kinases (in this case, cellular enzymes).[9] Ribavirin triphosphate (RTP) inhibits not only viral RNA polymerases[10] but also

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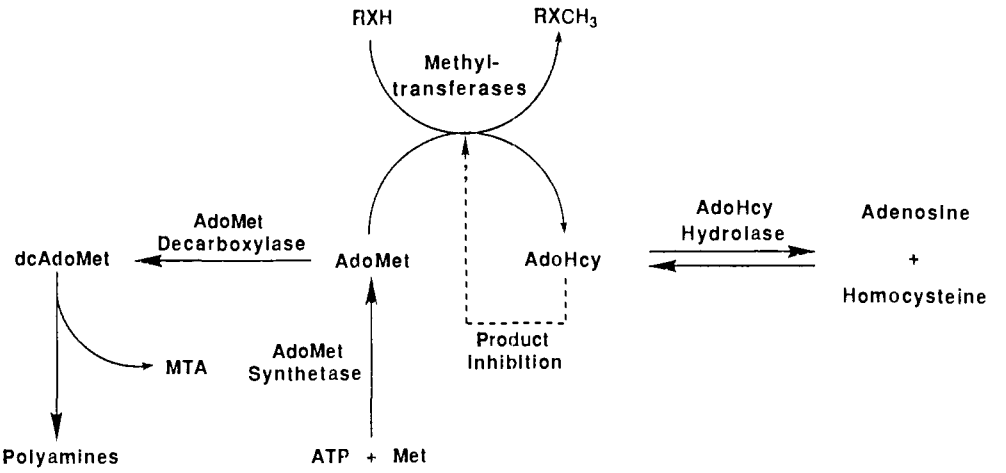
Scheme I. The Role of AdoHcy Hydrolase in Regulating AdoMet Dependent Biological Methylation



the virus-specific mRNA capping enzyme guanylyltransferase.[11] A eukaryotic mRNA must possess a methylated 5′-cap structure for stability against phosphatases and ribonucleases, for proper binding to ribosomes, and for the promotion of splicing;[12-14] hence, an uncapped mRNA is much less likely to be translated into its respective protein. This capping involves the transfer of the guanylate portion of guanosine triphosphate (GTP) to the 5′-end of the mRNA, forming an unusual 5′-5′-triphosphate linkage. The guanosine cap and penultimate nucleotide residue are subsequently methylated by specific methyltransferases. Since many types of viruses also require methylated 5′-capped mRNA for proper translation into proteins,[12,15] interference with the formation of these 5′-caps (e.g., through the inhibition of guanyltransferase) could conceivably lead to inhibition of viral replication.

Ribavirin 5′-monophosphate (RMP) is also thought to be involved in the antiviral action of the parent compound. RMP serves as an inhibitor of inosine monophosphate (IMP) dehydrogenase.[16] This enzyme catalyzes the conversion of IMP to xanthate monophosphate (XMP), the first specific step in the de novo synthesis of GMP. The inhibition of IMP dehydrogenase results in a marked reduction of intracellular GTP.[17] Since GTP competes with RTP for sites on the viral RNA polymerase and guanylyltransferase, lower levels of GTP would be expected to potentiate the antiviral effect of RTP. [Further evidence for the importance of IMP dehydrogenase inhibition in ribavirin's therapeutic effect: ribavirin's antiviral activity is reversed in cell culture by the addition of guanosine, but to a significantly lesser extent by inosine.[16] However, the depletion of GTP levels is probably not its sole mechanism of action, since it does not explain this drug's lack of effectiveness against certain viruses (*all* viruses require GTP for replication).] A total shut down of GTP synthesis would undoubtedly lead to cell death, but apparently partial inhibition is beneficial in this case. So the current dogma does have its exceptions: interference with normal cellular metabolism does not preclude a clinically useful antiviral agent.

II. Concept: *S*-Adenosyl-L-homocysteine Hydrolase Inhibition and Antiviral Activity

In fact, inhibition of a cellular enzyme is apparently the *basis* for the antiviral activity of certain adenosine analogues.[18a] A good correlation exists between the antiviral effectiveness of these adenosine analogues and their ability to inhibit *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1). De Clercq and Cools found a linear relationship between the log IC_{50} values (concentration which inhibits vaccinia virus replication by 50%) for a series of such adenosine analogues and their log K_i values for inhibition of murine L929 cell AdoHcy hydrolase.[19] Similar results were obtained with log IC_{50} values versus vesicular stomatitis virus.

AdoHcy hydrolase inhibitors are potent as well as broad-spectrum antiviral agents, inhibiting the replication of a variety of (−)RNA viruses (including such clinically important viruses as measles, respiratory syncytial virus, influenza A and B, and rabies) and double-stranded RNA viruses (including rotavirus, a major cause of gastroenteritis in children).[18] They are not particularly active against (+)RNA viruses or DNA viruses (except for vaccinia and African swine fever viruses), nor are they active against HIV.[18a,20] (The fact that the various AdoHcy hydrolase inhibitors affect the same set of viruses is another indication that this inhibition is their common mechanism of action.) Their wide range of activity is in contrast to almost all clinically used nucleoside antiviral drugs, which are usually specific toward a particular species or strain of virus. The paucity of broad-spectrum antiviral agents greatly limits the ability of the physician to treat viral diseases. This is further complicated by the difficulties involved in identifying a viral pathogen infecting a patient. Quite often there is simply no time for such identification: immediate action is necessary lest the patient's condition

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become much worse. The need for broad-spectrum antivirals is clear; the path toward their development is not. Interference with a normal cellular process which is required for viral replication could be one such path, but that process must be interrupted only partially and/or for a short period of time to avoid unwanted toxicity. It is this possibility which makes the broad-spectrum antiviral activity of AdoHcy hydrolase inhibitors so intriguing.

In this Perspective, we intend to analyze the therapeutic potential of AdoHcy hydrolase inhibition with respect to antiviral activity, providing background information as necessary. Our purpose is not to provide a rigorous review of the vast primary literature concerning AdoHcy hydrolase and its inhibitors (see refs 18a, 21, and 22 for recent reviews), but rather to explain why AdoHcy hydrolase has become such an attractive target for the design of antiviral agents, to describe how selective inhibitors with improved antiviral effectiveness were developed and how these inhibitors have led to a better understanding of the regulatory role of AdoHcy hydrolase in biological methylations, and, finally, to project the direction in which this area of research is headed.

The normal cellular role of AdoHcy hydrolase is regulating *S*-adenosyl-L-methionine (AdoMet) dependent biological methylation reactions (Scheme I).[21] AdoMet is involved in the methylation of many biomolecules, from small molecular weight neurotransmitters (e.g., histamine, norepinephrine) to macromolecules (e.g., proteins, lipids, nucleic acids), and the various methyltransferases which catalyze these reactions have themselves been targets for drug design.[23,24] [AdoMet is also decarboxylated by AdoMet decarboxylase to dcAdoMet. dcAdoMet serves as an aminopropyl donor toward the synthesis of polyamines and in the process is converted to 5′-methylthioadenosine (MTA). See Scheme I.] The byproduct of these methylations is AdoHcy, which functions as a feedback inhibitor of these methyltransferases. AdoHcy hydrolase provides the only known mechanism for AdoHcy catabolism in eukaryotes, catalyzing its hydrolysis to adenosine and homocysteine. Although the in vitro reaction favors the synthetic direction, subsequent metabolic conversions of adenosine (e.g., to ATP, inosine) and homocysteine (e.g., to methionine, cystathionine) within the cell assure the reaction will run in the hydrolytic direction.[25]

The mechanism by which the hydrolase catalyzes this reaction has been determined by Palmer and Abeles.[26] Mammalian AdoHcy hydrolase is a tetramer containing tightly (but not covalently) bound NAD^+. (The equivalence of the subunits has been a point of dispute in the literature,[22,27-30] the controversy fueled by conflicting evidence concerning the number of moles of NAD^+ and NADH per mole of enzyme tetramer.[26,27,31-35]) The first step in the enzymatic reaction is oxidation of the 3′-hydroxyl group of AdoHcy to a ketone; NAD^+ is converted to NADH in the process. A basic residue in the active site then removes the now more acidic 4′-proton, and elimination of homocysteine follows. (Attempts to determine the type of residue responsible for the 4′-proton removal have been inconclusive.[36-38]) Water then adds in a Michael fashion to the α,β-unsaturated ketone intermediate, and the NADH reduces the 3′-ketone back to a hydroxyl group. The newly formed adenosine then dissociates from the enzyme, which is now ready for another round of catalysis. Other mechanistic studies revealed that the 3′-oxidation is not the sole rate-determining step[39] and that the overall course of the reaction runs with retention of the C5′ configuration.[40]

The increased cellular AdoHcy levels that result from AdoHcy hydrolase inhibition have been correlated with the antiviral activity of these inhibitors.[41,42] [The concomitant decrease in homocysteine levels is not thought to be responsible for the antiviral activity since exogenous homocysteine potentiates this effect (vide infra).] AdoHcy, as mentioned before, is a feedback inhibitor of AdoMet-dependent methyltransferases,[43] and this includes vaccinia virus mRNA guanine-7- and nucleoside 2′-*O*-methyltransferases, which are involved in the formation of the vaccinia virus 5′-cap structure. Two important questions needed to be addressed: how might increased AdoHcy levels lead to inhibition of viral replication, and can interference with such an important cellular regulatory enzyme like AdoHcy hydrolase result in antiviral activity without causing undue host cell toxicity?

Toward the first question, it is suspected that the increased levels of AdoHcy interfere with the action of certain methyltransferases crucial to viral replication. No viral genomes are known to code for AdoHcy hydrolase, but several viruses sensitive to AdoHcy hydrolase inhibitors (e.g., vaccinia,[44] reovirus[45]) express their own specific 5′-cap mRNA methyltransferases. In fact, undermethylation at the 5′-terminus of viral mRNA induced by inhibition of cellular AdoHcy hydrolase has been correlated with inhibition of viral replication.[46] Selective

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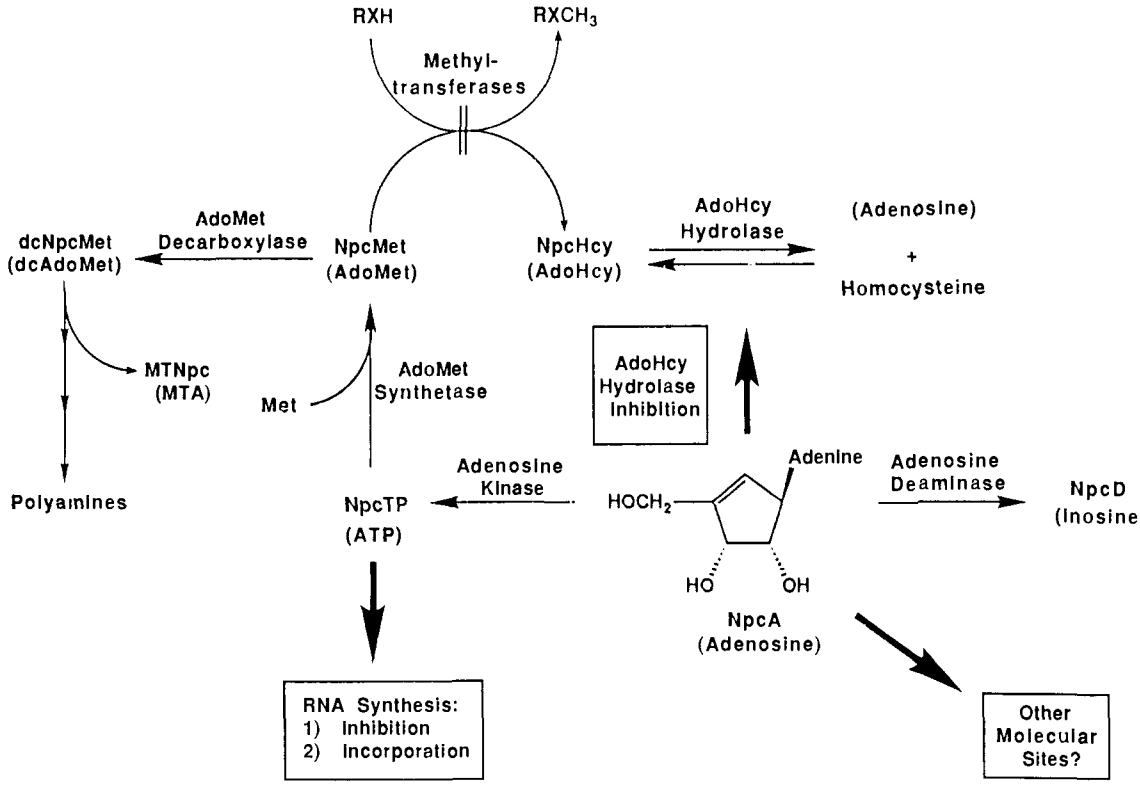
Scheme II. Metabolic Pathways of NpcA[a]



[a] The normal adenosine metabolites are shown in parentheses.

inhibition of this type of methylation might result from differences in the K_i of AdoHcy and the K_m of AdoMet toward the various methyltransferases. Hence, virus-specific 5′-cap methyltransferases may be more sensitive to rises in intracellular levels of AdoHcy than cellular methyltransferases.

On the other hand, selective inhibition of viral methyltransferases need not be invoked to explain the observed antiviral activity of AdoHcy hydrolase inhibitors: a *general* inhibition of 5′-cap mRNA methylation might be operative. Such a general inhibition of cellular RNA cap methylation would be expected to lead to a general suppression of protein synthesis and subsequent host cell toxicity, but if virus-infected cells have a higher overall rate of protein synthesis, these cells may be more sensitive to changes in AdoHcy levels than uninfected cells. This is supported by the fact that infection of murine L-929 cells with vaccinia causes a large increase in AdoHcy hydrolase activity.[47] This increased activity may be necessary to sustain viral replication. Also, the cell already has on hand a considerable supply of proteins needed to carry out its myriad functions, and so a slowdown in protein synthesis may be more detrimental to viral replication than to cellular function. (Besides, the synthesis of new cellular proteins has already been comprised by the viral infection.) Of course, a complete and/or long-term inhibition of protein synthesis would result in cell death or deleterious changes in cell morphology, but a temporary and partial inhibition, while not seriously altering cell function, may allow phosphatases and ribonucleases to destroy the foreign mRNAs. After removal of the AdoHcy hydrolase inhibitor, cellular mRNA cap methylation would resume, and full protein synthesis would ensue.

To summarize up to this point, the interest in AdoHcy hydrolase as a target for the design of antiviral agents has arisen because (i) most plant and animal viruses require a methylated cap structure at the 5′-terminus of their mRNA for viral replication,[12,15] (ii) virus-encoded methyltransferases that are involved in the formation of this methylated cap structure are inhibited by AdoHcy,[43] (iii) undermethylation of the viral mRNA cap structure induced by the inhibition of AdoHcy hydrolase has been correlated with the inhibition of viral replication,[46] (iv) a close correlation exists between the antiviral potency of adenosine analogues and their inhibitory effects on AdoHcy hydrolase,[19] and (v) a close correlation exists between the antiviral potency of carbocyclic nucleosides and their ability to elevate cellular levels of AdoHcy.[41,42]

III. Emergence of Selective AdoHcy Hydrolase Inhibitors

More direct proof concerning the mechanism of antiviral activity of these compounds was sorely lacking, mainly due to the absence of the necessary molecular tools: selective inhibitors of AdoHcy hydrolase. A number of strong inhibitors had been identified, and their broad-spectrum antiviral potencies elicited the concept of the hydrolase as a chemotherapeutic target. These first-generation hydrolase inhibitors (Figure 1) included adenosine analogues with acyclic "sugar" moieties such as 9(*S*)-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA],[48-51] D-eritadenine,[51-54]

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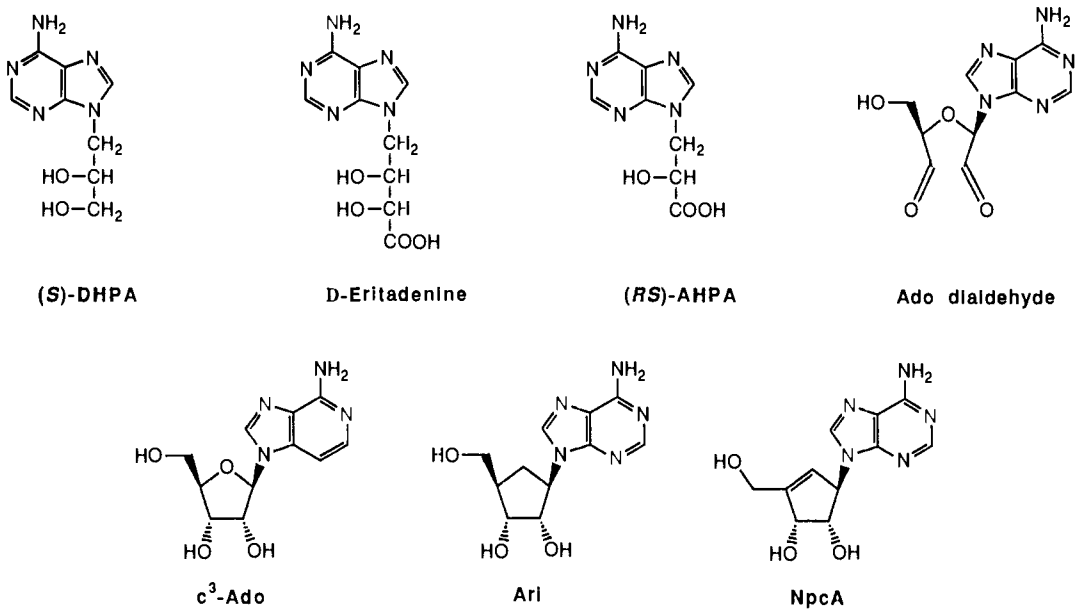

Figure 1. First-generation AdoHcy hydrolase inhibitors.

(*R,S*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*R,S*)-AHPA],[55,56] and adenosine dialdehyde.[57,58] Adenosine analogues with ribose or carbocyclic ribose moieties were also effective inhibitors: 3-deazaadenosine (c^3-Ado),[59-63] and the carbocyclic nucleosides aristeromycin (Ari)[63,64] and neplanocin A (NpcA).[47,65,66] Some of these compounds are quite potent, with K_i values for AdoHcy hydrolase below 10 nM; unfortunately, the cytotoxicity of these compounds precluded clinical use as antiviral agents. Was their toxicity due to inhibition of AdoHcy hydrolase or due to other interactions?

Clues concerning the nature of this toxicity were provided by cellular metabolism studies on NpcA (Scheme II) and Ari. A concentration as low as 0.1 μM NpcA inactivates 90% of the hydrolase in mouse L929 fibroblastoma cells, and a marked increase in intracellular AdoHcy/AdoMet levels results.[47] While not a substrate for the hydrolase, NpcA is converted by adenosine deaminase to the biologically inactive neplanocin D, the cyclopentenyl counterpart of inosine.[67,68] However, this metabolic route does not seem to be important since coadministration of deoxycoformycin or EHNA (both adenosine deaminase inhibitors) did not potentiate NpcA's effects in several different cell lines.[68-70]

NpcA is also converted to its corresponding 5'-triphosphate (NpcTP), presumably via adenosine kinase and, subsequently, adenylate kinase and nucleoside diphosphokinase.[69-71] The AdoMet counterpart of NpcA (NpcMet) is formed as well,[70-74] 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,[75] and in murine leukemia cells, a NpcA-resistant cell line possessed decreased adenosine kinase activity, suggesting that 5'-phosphorylation leads to the antitumor activity observed in the normal cell line.[76] On the other hand, Chinese hamster ovary (CHO) cells are known to metabolize NpcA to NpcTP, but an adenosine kinase mutant ($AdoK^-$) cell line (which produced little, if any, NpcTP) was only slightly more resistant to NpcA treatment.[69] Not much is known about NpcTP's mechanism of toxicity, except

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that it is converted to NpcMet and is minimally incorporated into RNA,[70] but given the ubiquity of ATP's involvement in biological processes, 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 et al.[70,75] suggest that it may inhibit cellular RNA methylation (HT-29 cells) and that NpcA may exert its toxic effect through this metabolite. To date, NpcMet's effect on mRNA methyltransferases 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.[68] NpcMet is also neither substrate nor inhibitor of AdoMet decarboxylase;[68] however, it did serve as a substrate for catechol-*O*-methyltransferase.[73] 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.

Ari's metabolism is somewhat similar to that of NpcA. Kinases also metabolize Ari to its 5′-phosphates,[77] and these nucleotides have been implicated with cellular toxicity.[78,79] However, Ari apparently kills cells by different mechanisms in adenosine kinase deficient ($AdoK^-$) and normal ($AdoK^+$) cell lines.[80] In $AdoK^+$ cells, the phosphate metabolites of Ari are presumably responsible for the toxicity, while in $AdoK^-$ cells, AdoHcy hydrolase inhibition may be the cause. Overinhibition of AdoHcy hydrolase has been implicated with cytotoxicity, and this could be due, at least in part, to homocysteine depletion.[81,82] The hydrolysis of AdoHcy is the only source of homocysteine in mammalian cells,[83] and homocysteine, by conversion to methionine, helps regenerate tetrahydrofolate needed for purine and pyrimidine de novo synthesis.[84] Hence, a depletion of homocysteine results in an inhibition of nucleic acid biosynthesis. On the other hand, the importance of this homocysteine depletion in the cytotoxicity of hydrolase inhibitors can be judged by the observation that, contrary to expectation, cotreatment of these inhibitors with homocysteine *potentiated* their cytotoxic effect (vide infra).[85-87]

Another Ari metabolite is a possible cause of toxicity: carbocyclic GMP. Ari's 5′-monophosphate serves as a substrate for AMP deaminase, converting it to the IMP analogue of Ari.[79,88] This metabolite is then transformed to phosphates of carbocyclic guanosine. The carbocyclic analogue of GMP is a good inhibitor of hypoxanthine-(guanine) phosphoribosyltransferase,[79] an important enzyme in the purine salvage pathway. This explains the complete blockade of the utilization of hypoxanthine and guanine upon Ari treatment.[78,79]

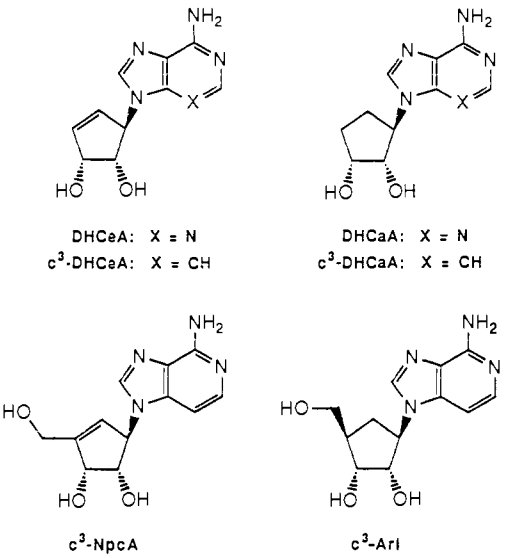

Figure 2. Second-generation AdoHcy hydrolase inhibitors.

Is the antiviral activity of Ari and NpcA the result of increased AdoHcy levels or the result of the formation of these metabolites (or a combination of these)? Again, selective AdoHcy hydrolase inhibitors would help answer this question. Clearly, removal of the hydroxymethyl substituent would preclude 5′-phosphorylation by adenosine kinase. SAR studies on adenosine deaminase indicated that the 5′-hydroxyl is essential for substrate activity toward this enzyme as well.[89] The resultant analogue of NpcA (dihydroxycyclopentenyladenine, DHCeA; Figure 2) and its 3-deaza counterpart (c^3-DHCeA), synthesized in our laboratory,[90] did not in fact serve as a substrate for these two enzymes;[91] however, they did retain potent inhibitory activity against AdoHcy hydrolase.[92] Their saturated counterparts (DHCaA and c^3-DHCaA) also proved to be potent and selective hydrolase inhibitors.[93] Using similar reasoning, Marquez et al. designed and synthesized c^3-NpcA.[75,94] This compound, which also lacks substrate activity toward adenosine kinase and adenosine deaminase, is the most potent hydrolase inhibitor to date. Similar results have been observed with c^3-Ari.[95]

These second generation AdoHcy hydrolase inhibitors retain antiviral activity while their cytotoxicity is considerably lower than the parent compounds.[20,94-96] NpcA is

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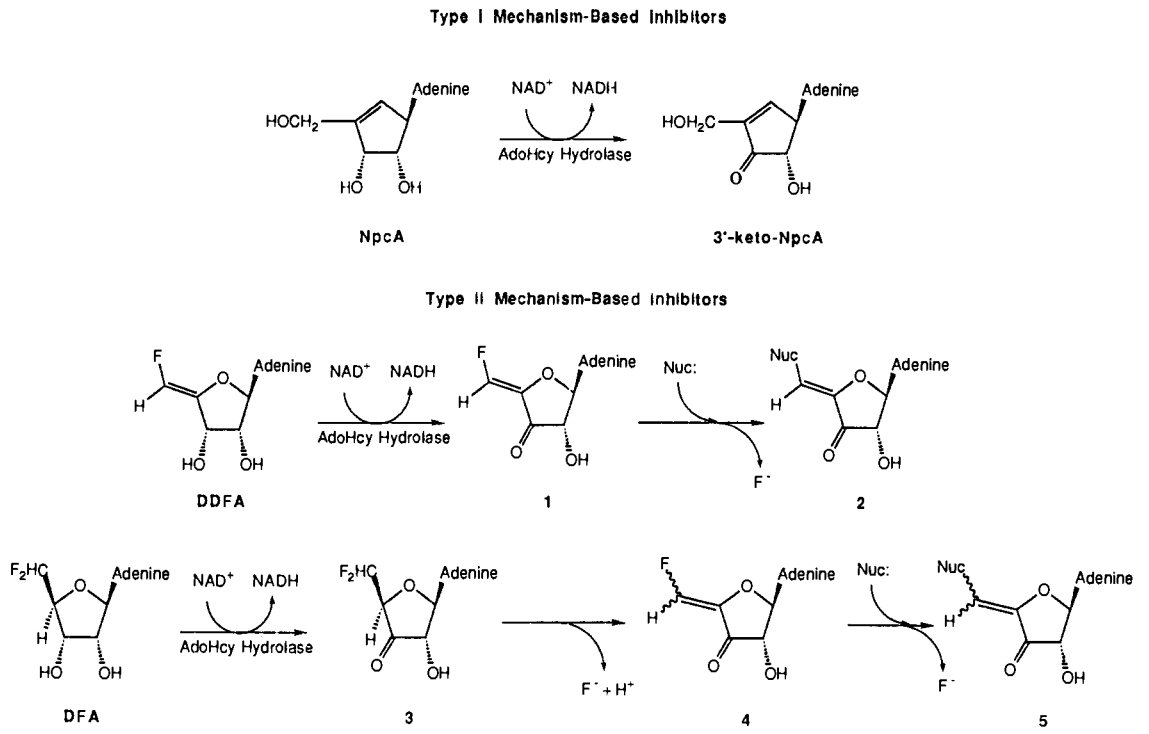
Scheme III. Classification of AdoHcy Hydrolase Inactivators



a more potent inhibitor of vaccinia virus replication (IC_{50}) in murine L929 fibroblast cells than DHCeA by a factor of 3; however, DHCeA is 34 times less cytotoxic (measured as the ID_{50} value, the concentration of drug which causes the inhibition of 50% cellular replication). Using ID_{50}/IC_{50} as a measure of therapeutic effectiveness, DHCeA is therefore a better antiviral agent than NpcA by a factor of 10. By this criteria, the Ari analogues DHCaA and c^3-DHCaA were also much better antiviral agents than their parent compound by factors of 330 and 670, respectively.[97] Apparently, elimination of adenosine kinase and deaminase substrate activity led to the reduced toxicity of these analogues.

An understanding of the mechanism by which these compounds inhibit the hydrolase would assist in the design of "new and improved" inhibitors that are more potent but which retain the selectivity described above. Studies with tritiated NpcA[98] showed that this compound is oxidized in the 3′-position as is adenosine; in the process, NADH is converted to NAD^+. However, the reaction stops at this point since NpcA has no 4′-proton for removal. The 3′-keto-NpcA has been isolated from inactivated hydrolase under mild denaturing conditions (i.e., it is a stable species and remains tightly bound to the NADH form of the enzyme). Hence, NpcA inactivates the hydrolase by a "cofactor-depletion mechanism", converting the NAD^+ cofactor to its inactive form (NADH). We call compounds which inactivate the enzyme in this way type I mechanism-based inactivators (i.e., compounds causing irreversible cofactor depletion but which are not covalently bound to the enzyme; Scheme III). DHCeA is another example of a type I mechanism-based inactivator.[99] [The possibility remains that the 3′-keto derivatives of NpcA and DHCeA are attacked by enzyme nucleophiles and that this covalent binding is reversed by the denaturation conditions. This would relegate these two inactivators to the type II division (vide infra; Scheme III). However, their saturated counterparts, Ari and DHCaA, also inactivate the enzyme, presumably with concomitant conversion of NAD^+ to NADH.[93] The putative saturated 3′-keto intermediates that would result cannot be Michael acceptors, so covalent binding is not likely.] Ari analogues potentially capable of forming 3′-keto-NpcA in the active site of AdoHcy hydrolase (e.g., 6′-β-fluoroaristeromycin) have also been described as potent inhibitors[100] and may be type I inactivators as well.

Type II mechanism-based inactivators are those that are not only oxidized in the 3′-position by the hydrolase, but also become covalently bound to the enzyme (Scheme III). Investigations by McCarthy et al.[101] indicate that 4′,5′-didehydro-5′-fluoroadenosine (DDFA) might be such an inactivator. This compound causes the reduction of hydrolase NAD^+ to NADH as do type I inactivators; however, fluoride is also released quantitatively in the presence of the enzyme, suggesting that the 3′-keto form of this molecule might be attacked by an enzyme nucleophile. 5′-deoxy-5′-difluoroadenosine (DFA) is another example of a potential type II inactivator.[102] This compound, in the presence of the hydrolase, releases *two* fluoride ions per mole inactivator, conceivably due to elimination of fluoride ion from the 3′-keto intermediate followed by nucleophilic attack on the vinyl fluoride so formed. (It is possible that enzyme-sequestered water is the nucleophile in both cases, and this would consign these inactivators to the type I division.)

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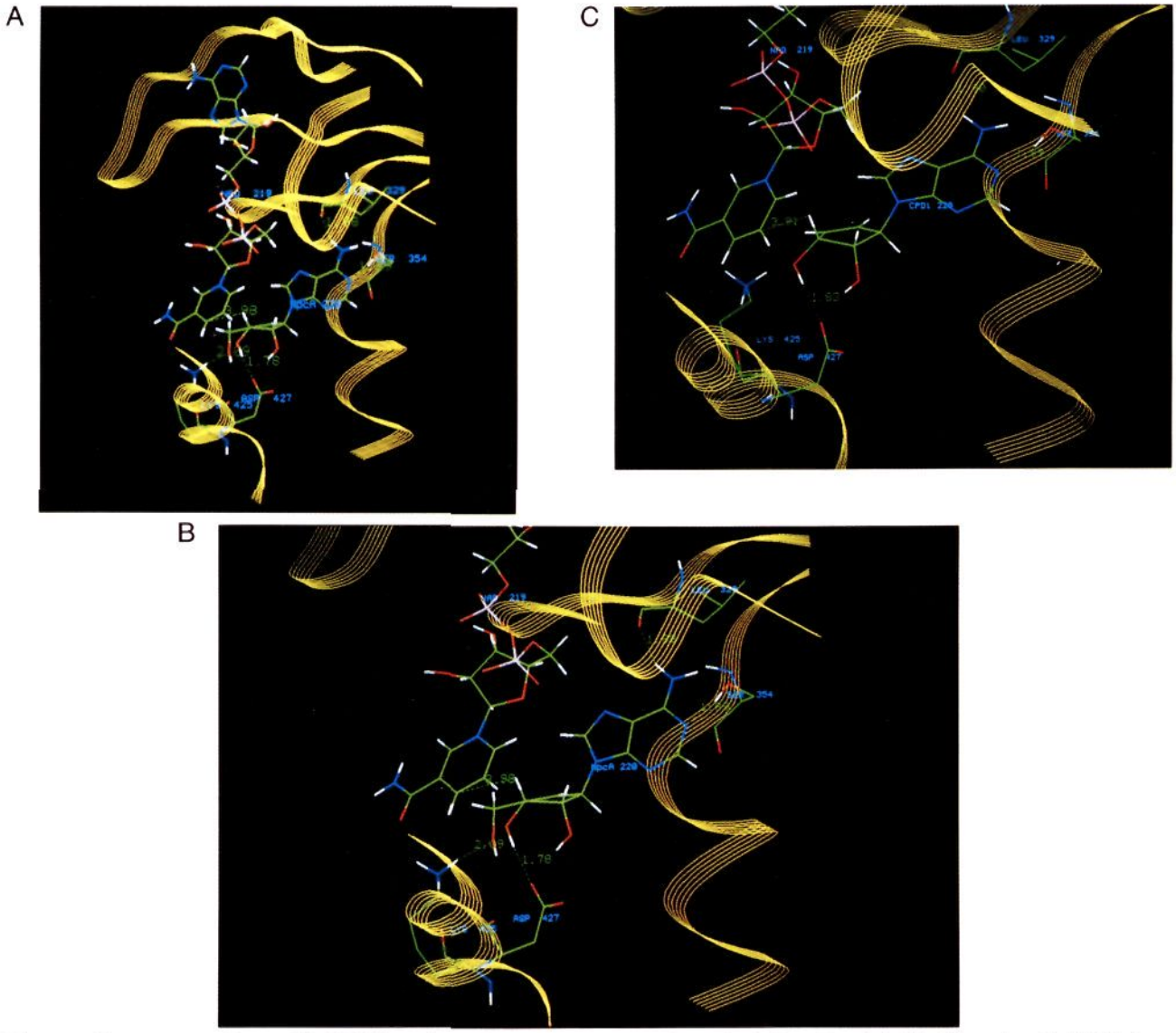

Figure 3. Computer-graphics model of inhibitors docked in the active site of AdoHcy hydrolase. Panel A: NpcA bound to the NAD^+-form of the enzyme. Protein backbone (yellow ribbon), NAD^+, and select side chains shown. Panel B: Closeup of top panel. Note the proximity of the 3′-hydrogen of NpcA to the nicotinamide moiety of NAD^+. Hydrogen bonding between NpcA and key residues depicted may be responsible for the high affinity of this adenosine analogue to the enzyme. In particular, note the hydrogen bonds between NpcA's 5′-hydroxyl group and Lys-425, NpcA's 3′-hydroxyl group and Asp-427, and NpcA's amino group and the backbone carbonyl of Leu-329. Panel C: NpcA analogue DHCeA (labeled as Cmpd1) bound to the active site. The lack of a 5′-hydroxyl group to hydrogen bond to Lys-425 is thought to be the cause of the reduced affinity of this analogue for the enzyme compared with that of the parent compound.

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 led to adenosine analogues with potent broad-spectrum antiviral activity but with reduced cytotoxicity in comparison to less selective analogues. With these molecular tools, it is likely that the biochemical mechanism by which AdoHcy hydrolase inhibition causes antiviral effects will soon be elucidated.

IV. Toward Third-Generation Hydrolase Inhibitors

New techniques in molecular biology and computational chemistry are being utilized in order to design even more potent hydrolase inhibitors which retain the selectivity of the second-generation inhibitors. The cloning of AdoHcy hydrolase from rat liver,[29] human placenta,[103] and the primitive eukaryote *Dictyostelium discoideum*[104] has allowed for the relatively facile determination of their respective amino acid sequences. The remarkable homology between the hydrolase primary sequences of these three species suggests that the hydrolase has been well-conserved in evolution. Furthermore, these clones also contain a "fingerprint" sequence characteristic of dinucleotide-binding domains of many proteins.[105] Site-directed mutagenesis in this sequence results in drastic changes in the NAD^+ binding affinity and the catalytic capability of the recombinant rat liver enzyme. Three glycine residues found in this sequence were each separately mutated to

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valine, resulting in mutant proteins which did not bind NAD^+, were catalytically inactive, and existed as monomers.[106] The mutation of Asp-244 (also in the hypothesized NAD^+ binding domain) to Glu resulted in an enzyme with reduced affinity for NAD^+, while retaining K_m values for substrates that are comparable to those of the wild-type enzyme, strongly suggesting that this residue is involved in NAD^+ binding.[107]

The determination of the amino acid sequence of the hydrolase from these clones paved the way for the development of computer graphics models 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) contain 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.[108,109] 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[110] (Figure 3) by computational mutation of LDH, a functionally similar (i.e., NAD^+-binding) protein of known structure. Ari and NpcA and a number of their analogues were examined with this model, and the calculated binding strengths of these inhibitors correlated with their observed K_i values for purified AdoHcy hydrolase. Thus, the relative binding energies of these inhibitors with the model were used to obtain a linear relationship with their log K_i values. This model has proven useful in predicting structures that would have either high or low affinity for the active site of this enzyme.[110] In turn, additional inhibition studies are being used to adjust the initial model.

Molecular biological techniques may 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.[85,111,112] This could be explained by the existence of separate "pools" of the hydrolase, some of which may be inaccessible to adenosine analogues; however, two isoforms of the enzyme, one inhibitor-sensitive and the other inhibitor-insensitive, have been separated by chromatographic means.[113] These isozymes 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.[66] A given nucleoside analogue might be less effective in preventing viral infection in those cell lines which possess different proportions of the inhibitor-insensitive isozyme. (The observed differences in sensitivity of particular cell lines to AdoHcy hydrolase inhibition might also be explained by differences in AdoHcy export and variable levels of AdoMet.[114]) From a drug-development standpoint, knowledge of the structural character of the isozymes might assist in the design of compounds capable of inhibiting the hydrolase in both (or all) of its forms. Thus, lower concentrations of inhibitor would be needed to attain the same AdoHcy/AdoMet ratios and, by our hypothesis, the same antiviral activity as that caused by higher concentrations of hydrolase inhibitors which only affected one isoform. Clones, site-directed mutants, computer models, monoclonal antibodies: all these tools of molecular biology can be utilized to arrive at an understanding of the three-dimensional structure(s) of the inhibitor-insensitive form(s) of the enzyme.

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 isoforms the result of different genes, alternate mRNA splicing or posttranslational modification of a single gene product? How is the expression of the different isoforms regulated? What are the sources of AdoHcy in the cell?

The answer to this last question appears to be not only AdoMet (via methyltransferases) but also adenosine and homocysteine (via AdoHcy hydrolase). Double-labeling experiments using [^{3}H]adenosine and [^{35}S]methionine showed that the ratio [^{3}H]/[^{35}S] was higher in AdoHcy than AdoMet in cells inhibited with selective hydrolase inhibitor DHCeA, suggesting that the inhibitor-insensitive form of AdoHcy hydrolase contributes to the biosynthesis of AdoHcy. This knowledge is not of academic interest alone. Homocysteine potentiates the antiviral effects of hydrolase inhibitors, and the above information suggests that this synergistic effect may be due to this amino acid's conversion to AdoHcy via the hydrolase. In fact, with hydrolase inhibitor and homocysteine, increases in cellular AdoHcy/AdoMet to levels unattainable with drug alone have been observed. Unfortunately, the additional homocysteine also potentiates the cytotoxic effects of these compounds (vide supra); however, the antiviral potentiation is greater. The overall result is an increase in the therapeutic effectiveness (ID_{50}/IC_{50}) of the adenosine analogue.[85,87]

The main objection to this strategy toward the development of antiviral agents is that there is intrinsic toxicity associated with inhibiting such a key regulator of cellular function. This is certainly a valid point, 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 and c^3-NpcA, the intrinsic toxicity of hydrolase inhibition might be determined.

How could this intrinsic toxicity be determined? The use of selective AdoHcy hydrolase inhibitors led to the discovery of a relationship between the ratio AdoHcy/AdoMet and cellular toxicity. This ratio has been determined at the ID_{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 IC_{50} values revealed that the ratio is consistently 0.15–0.20 (Figure 4).[41]

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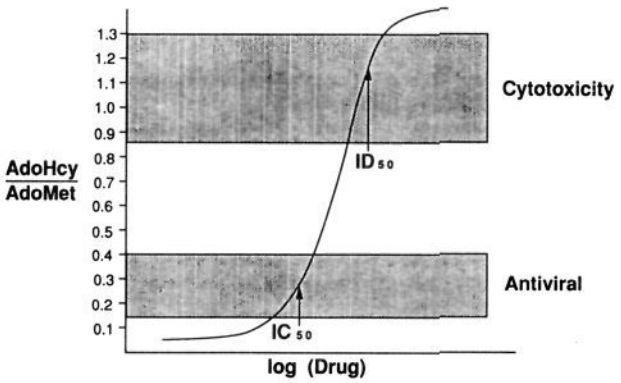

Figure 4. The relationship between drug concentration and the ratio AdoHcy/AdoMet in murine L929 cells. Antiviral effects are observed at lower AdoHcy/AdoMet levels than cytotoxic effects. [IC_{50} = drug concentration resulting in 50% inhibition of viral replication. ID_{50} = drug concentration causing 50% inhibition of cellular replication.] The abscissa has no units because different drugs require different concentrations for the same AdoHcy/AdoMet ratio; however, the concentrations are generally in the micromolar range.

These results indicate what we already knew: that it is possible to achieve antiviral effectiveness by inhibiting AdoHcy hydrolase while keeping the cytotoxicity to a minimum.

Any excitement generated by these data is tempered by a problem which arises from them: the difference between the IC_{50} and ID_{50} values for hydrolase inhibitors has not reached 3 orders of magnitude.[97] If the cytotoxicity of these compounds is due solely to hydrolase inhibition (and this may not be so), then the relative difference between the IC_{50} and the ID_{50} cannot be improved: by designing and synthesizing a more potent hydrolase inhibitor, the ID_{50} value would be expected to drop along with the IC_{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. Homocysteine is one such compound, mentioned earlier. 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.[115] 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.[116] 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 (vide supra). When the antiviral activity (vaccinia virus; murine L-929 cells) of c^3-DHCeA was determined in the presence of ribavirin, the antiviral effectiveness (ID_{50}/IC_{50}) of c^3-DHCeA increased 10-fold.[117] 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.

The development of third-generation AdoHcy hydrolase inhibitors will rely heavily on new techniques in molecular biology and computational chemistry. These techniques will aid the medicinal chemist in designing very potent inhibitors which retain selectivity for AdoHcy hydrolase. In addition, the discovery that homocysteine and 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 adenosine analogues in clinical situations.

V. Conclusions

The diverse means by which the various viruses infect cells and their remarkable mutation rate defies a simple unifying mechanism for their eradication. Nevertheless, compounds such as ribavirin and AdoHcy hydrolase inhibitors indicate that the development of broad-spectrum nucleoside antiviral agents is possible. The main stumbling block to clinically useful AdoHcy hydrolase inhibitors is their cytotoxicity; however, through the design and synthesis of selective inhibitors, compounds of significantly reduced toxicity have been achieved. The question is how far can this toxicity be reduced while retaining antiviral activity. With the new molecular biological tools and the increased capabilities of computer modeling, the answer may soon be forthcoming. The utility of these agents in antiviral chemotherapy may be limited to combination regimens or to prophylaxis, but even if these studies 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.

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