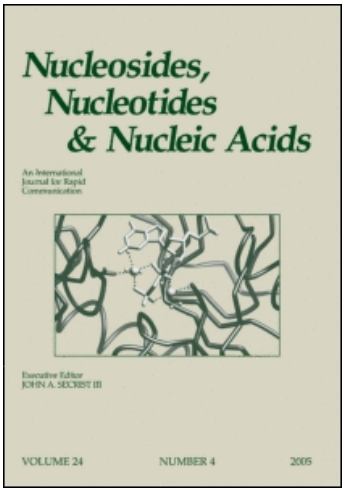

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Elucidation of the Mechanism by Which 9-(Trans-2', Trans-3'-Dihydroxycyclopent-4'-enyl)-Adenine Lnactivates S-Adenosylhomocysteine Hydrolase and Elevates Cellular Levels of Sadenosylhomocysteinew

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ELUCIDATION OF THE MECHANISM BY WHICH 9-(*TRANS*-2',*TRANS*-3'-DIHYDROXYCYCLOPENT-4'-ENYL)-ADENINE INACTIVATES S-ADENOSYLHOMOCYSTEINE HYDROLASE AND ELEVATES CELLULAR LEVELS OF S-ADENOSYLHOMOCYSTEINE

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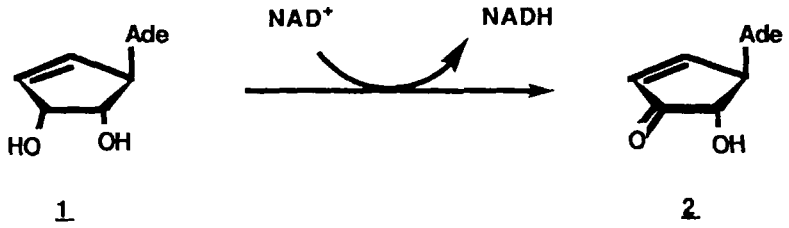
Abstract. Evidence is presented that 9-(*trans*-2',*trans*-3'-dihydroxycyclopent-4'-enyl)-adenine (1) is oxidized to the 3'-ketonucleoside 2 with concomitant reduction of NAD^+ to NADH by S-adenosylhomocysteine hydrolase. We also describe how the carbocyclic nucleoside 1 has been used to reveal the metabolic relationships between S-adenosylmethionine, S-adenosylhomocysteine and homocysteine in murine L-929 cells.

In recent years S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) has emerged as a target for the design of antiviral agents.[1] De Clercq and Cools[2] have established a close correlation between the antiviral potency of several adenosine analogs, including neplanocin A (NpcA), and their selective inhibitory effects on AdoHcy hydrolase. Inhibition of AdoHcy hydrolase results in increased levels of intracellular AdoHcy, a product inhibitor of S-adenosylmethionine (AdoMet)-dependent methyltransferases.[3] Keller and Borchardt[4] have proposed that the antiviral activity of AdoHcy hydrolase inhibitors results from the inhibitory effects of the elevated cellular levels of AdoHcy on methyltransferases necessary for the maturation of viral mRNA.

Recently, our laboratory described the synthesis[5] of 9-(*trans*-2',*trans*-3'-dihydroxy-cyclopent-4'-enyl)-adenine (1), an analog of NpcA. The carbocyclic nucleoside 1, a potent inhibitor of AdoHcy hydrolase, which is devoid of substrate activity with adenosine kinase and adenosine deaminase,[6,7] has been shown to have antiviral activity (e.g., vaccinia virus) with reduced cytotoxicity (e.g., murine L-929 cells).[8] The carbocyclic nucleoside 1,[6] like 2'-deoxyadenosine[9] and NpcA[10] inhibits AdoHcy hydrolase by a mechanism involving reduction

of enzyme bound NAD^+ to NADH. Since this mechanism is analogous to the normal catalytic mechanism described by Palmer and Abeles,[11] it is assumed that reduction of NAD^+ to NADH is accompanied by the oxidation of the nucleoside to the 3'-ketonucleoside.

In this paper we provide direct evidence for the formation of the 3'-ketonucleoside 2 by AdoHcy hydrolase-catalyzed oxidation of the carbocyclic nucleoside 1 (Eq. 1). In addition, we show that the 3'-ketonucleoside 2 is a weak, reversible inhibitor of AdoHcy hydrolase, suggesting that the inactivation of AdoHcy hydrolase by compound 1 results simply from the irreversible reduction of NAD^+ to NADH. We also describe in this paper how compound 1 has been used to elucidate the interrelationships which exist in murine L-929 cells between the metabolism of AdoMet, AdoHcy and homocysteine (Hcy).



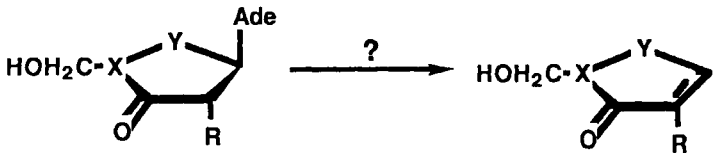
1 2 (1)

The Role of the 3'-Ketonucleoside 2 in the Inactivation of AdoHcy Hydrolase by Carbocyclic Nucleoside 1

The mechanism of inactivation of AdoHcy hydrolase by the carbocyclic nucleoside 1,[6] 2'-deoxyadenosine[9] and NpcA,[10] which involves the reduction of NAD^+ to NADH, assumes that the nucleosides are oxidized to the corresponding 3'-ketonucleosides. Abeles and coworkers have observed that adenine is released upon denaturation of the enzyme inactivated with 2'-deoxyadenosine or NpcA and, thus, suggest that the enzyme inactivation is irreversible as a consequence of elimination of adenine from the tightly bound 3'-ketonucleoside (Eq. 2), which prevents reoxidation of NADH to NAD^+.[9,12]

It has also been suggested that the keto sugar 3 resulting from the depurination of the 3'-ketonucleosides (Eq. 2) could act as a Michael acceptor and covalently modify the enzyme active site, suggesting a K_{cat} mechanism of inactivation.[9,12] However, direct evidence for the formation of the 3'-ketonucleosides and their roles, if any, in the mechanism of inactivation of AdoHcy hydrolase is minimal.

Therefore, in this study we have prepared the 3'-ketonucleoside 2 and studied its physicochemical properties and its ability to inhibit AdoHcy hydrolase. The 3'-



(2)

3

X-Y = CH-O, R = H (2'-deoxyadenosine)
X-Y = C=CH, R = OH (NpcA)

ketonucleoside 2 was prepared by the stoichiometric oxidation of 1 by bovine liver AdoHcy hydrolase (Fig. 1A) or by chemical oxidation of 1 with MnO_2. Enzymatic synthesis of 2 involved incubation of freshly prepared bovine liver AdoHcy hydrolase[13] (3 mg, 15 nmoles) with two equivalents of 1 for 60 min. at 37 °C followed by separation of the excess 1 by chromatography on Sephadex G-75. Earlier work from our own laboratory[6] showed that under these conditions, the NAD^+ bound to AdoHcy hydrolase was totally reduced to NADH. Adjusting the solution of inactivated enzyme to pH 2 with $HClO_4$ caused denaturation and precipitation of the protein. The protein was removed by filtration (Amicon PM30 membrane) and the filtrate concentrated. Analysis of the filtrate by HPLC (15 x 0.46 cm ODS Hypersil, 1 mL/min., A= 50 mM NaH_2PO_4 and 10 mM sodium heptanesulfonate adjusted to pH 3.2 with H_3PO_4, B= acetonitrile, gradient elution with 3-20% B over 25 min.) revealed the presence of a new peak (elution time 13.0 min.) and the absence of the carbocyclic nucleoside 1 (elution time 13.7 min., Fig. 1B). This new peak was not detectable when carbocyclic nucleoside 1 or AdoHcy hydrolase was acidified alone with $HClO_4$. This new peak, which is presumed to be the 3'-ketonucleoside 2, was isolated in pure form by preparative HPLC. Chemical oxidation of 1 with MnO_2 (Aldrich, "black activated") in DMSO (60 min. at 22 °C) afforded a product that co-eluted with the product generated by the AdoHcy hydrolase-catalyzed oxidation of 1. However, MnO_2 oxidation caused extensive degradation[14] of 1 (and/or 2) and thus the yield of the presumed 3'-ketonucleoside 2 was low. The oxidation product isolated from the AdoHcy hydrolase-catalyzed reaction or the MnO_2 oxidation reaction showed similar physicochemical properties. For example, the UV spectrum of the oxidation products had maxima at 214 and 260 nm in NaH_2PO_4 buffered to pH 3.2, as compared to 1 which had maxima at 204 and 260 nm. Neither method of preparation resulted in sufficient material for characterization by NMR spectroscopy, thus we resorted to chemical methods for definitive characterization of the oxidation product.

The product isolated from the AdoHcy hydrolase-catalyzed reaction and the MnO_2 oxidation reaction was shown to be the 3'-ketonucleoside 2 by reduction with sodium

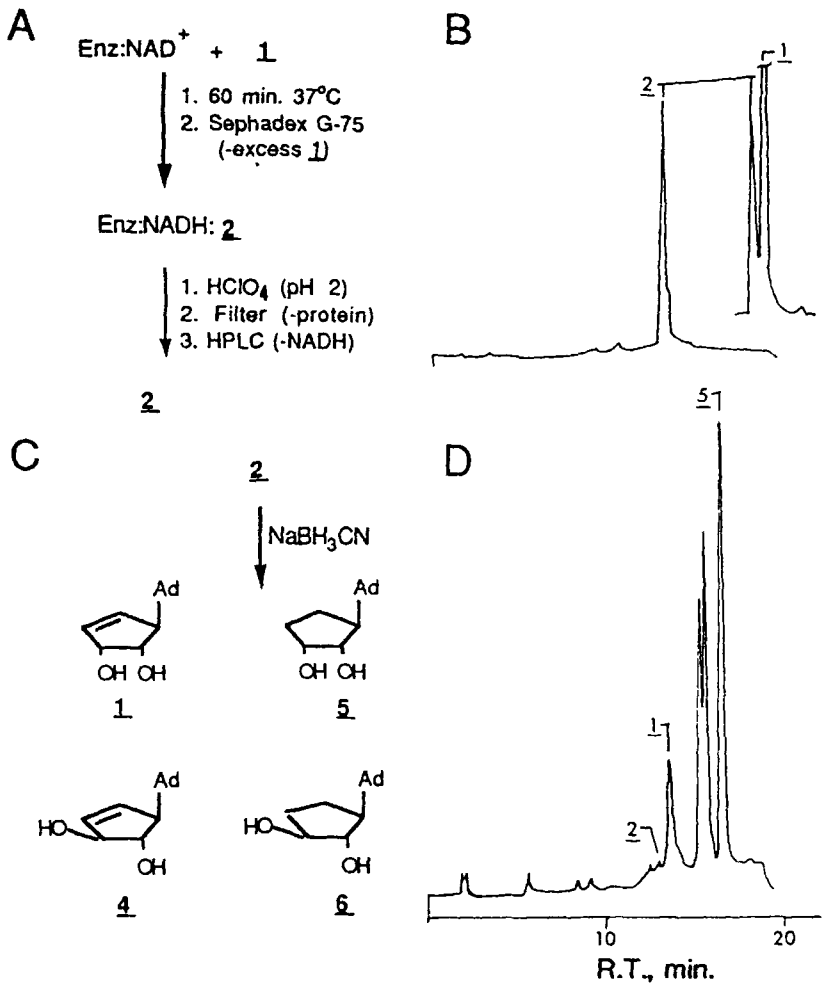


Figure 1. A) Scheme for enzymatic synthesis of 3'-ketonucleoside 2. B) HPLC chromatograms of 2 (inset, coinjection of 2 and 1). C) Products expected from $NaBH_3CN$ reduction of 2. D. Chromatogram of reduced 2, peaks labelled 1 and 5 were identified by coinjection with known samples. Elution conditions are described in the text.

cyanoborohydride ($NaBH_3CN$). $NaBH_3CN$ reduction of enones is not selective for 1,2 vs. 1,4 hydride addition. Therefore, reduction of 2 is expected to give a mixture of four possible products: 1, 9-(*trans*-2',*cis*-3'-dihydroxycyclopentenyl)-adenine (4), 9-(*trans*-2',*trans*-3'-dihydroxycyclopentyl)adenine (5), and 9-(*trans*-2',*cis*-3'-dihydroxycyclopentyl)-adenine (6) (Fig. 1C). Reduction (0.05 M $NaBH_3CN$, 22 °C, 4 hrs.) of the unknown product isolated from the AdoHcy hydrolase-catalyzed oxidation of 1 or the MnO_2 oxidation of 1, resulted in the

formation of four products (Fig. 1D). Two of these reduction products (elution times 13.7 and 17.0 min.) were confirmed to be compounds 1 and 5 by co-elution with authentic samples. The other two reduction products are presumed to be the *cis*-3'-hydroxy epimers 4 and 6, for which authentic samples are not available. These data confirm that the product generated by AdoHcy hydrolase-catalyzed oxidation of 1 is the corresponding 3'-ketonucleoside 2.

Since depurination of the 3'-ketonucleosides generated by AdoHcy hydrolase-catalyzed oxidation of 2'-deoxyadenosine and NpcA has been suggested to be a critical step in the irreversible inactivation of the enzyme,[9,12] we determined the chemical stability of the 3'-ketonucleoside 2. When 2 was incubated at 22 °C in pH 3.2 , pH 7.0, and pH 9.0 phosphate buffers, the first half-lives ($t_{1/2}$'s) for disappearance of the compound as determined by HPLC were 11 days, 72 hours, and 19 hours, respectively. The disappearance of the peak corresponding to 2 was linear with time, however because of limited quantities of 2 we were unable to distinguish between zero-order and first-order kinetics and we were unable to fully characterize the degradation products. However, only trace amounts of adenine were formed suggesting that depurination is a minor pathway of degradation of 2.

In an independent study,[15] we have prepared the 3'-keto derivative of NpcA and shown that it also is susceptible to base-induced degradation. In fact, the isolation of 3'-keto-NpcA from the AdoHcy hydrolase-catalyzed reaction was only possible when the inactivated enzyme was denatured with $HClO_4$ and the 3'-keto-NpcA purified and stored under acidic conditions. If the $HClO_4$ solution of the denatured protein and and 3'-keto-NpcA was neutralized as described by Wolfson et al.,[12] the 3'-keto-NpcA degraded rapidly to yield variable amounts of adenine as well as other products. The variable amounts of adenine being released from 3'-keto-NpcA may depend on the buffer remaining from gel filtration of the enzyme/3'-keto-NpcA adduct (e.g., tris buffers cause more significant release of adenine than phosphate buffers). In phosphate buffer 3'-keto-NpcA degrades at rates comparable to the 3'-ketonucleoside 2 and liberated only trace amounts of adenine. These results suggest that the depurination of the NpcA, which was proposed by Wolfson et al.[12] to be an integral step in the K_{cat} inactivation of AdoHcy hydrolase, is probably an artifact of the workup procedure.

In addition to determining the physicochemical properties of the 3'-ketonucleoside 2, we were also interested in determining its effects on the catalytic activity of AdoHcy hydrolase. Since 2 could only be isolated from the inactivated AdoHcy hydrolase by $HClO_4$ denaturation, it appears that the 3'-ketonucleoside 2 binds tightly to the NADH form of the enzyme. However, when the 3'-ketonucleoside 2 was evaluated as an inhibitor of the NAD^+ form of AdoHcy hydrolase, it was observed to be a weak, reversible inhibitor. The 3'-ketonucleoside 2

at concentrations up to 2 μM did not cause time-dependent inactivation of the NAD^+ form of the enzyme. Incubations were carried out at 37 °C up to 20 min. In contrast, compound 1 at a concentration of 2 μM produced complete, irreversible inactivation of the enzyme in 20 min. The 3'-ketonucleoside 2 was shown to be a weak, reversible inhibitor with a K_i = 0.8 μM as determined by Michaelis-Menton kinetics.[16] Similar results have been observed for 3'-ketoNpcA.[15]

The data presented above, as well as the observation by our laboratory that AdoHcy hydrolase inactivated by NpcA[10] or the carbocyclic nucleoside 1[6] can be reactivated by incubation with NAD^+, strongly suggest that these inhibitors inactivate the enzyme by a "cofactor depletion mechanism" involving simply conversion of the enzyme from the NAD^+ form (catalytically active) to the NADH form (catalytically inactive). These data do not support the K_{cat} mechanism proposed by Wolfson et al.[12] for the NpcA-induced inactivation of AdoHcy hydrolase.

The Use of Carbocyclic Nucleoside 1 to Elucidate the Interrelationships Between the Murine L-929 Cell Metabolism of AdoMet, AdoHcy and Hcy.

In an earlier study our laboratory showed that the carbocyclic nucleoside 1, at a concentration of 1.0 μM, produced rapid inactivation of murine L-929 cell AdoHcy hydrolase activity (within 30 min the enzyme was 95% inhibited).[7] This inhibition of AdoHcy hydrolase persisted for at least 72 hours, resulting in an increase in the intracellular level of AdoHcy and an increase in the ratio of AdoHcy/AdoMet.[7] Both of these parameters could be correlated with the ability of the drug to inhibit viral replication.[17]

More recently, we have studied in detail the concentration-dependent inactivation of L-929 cell AdoHcy hydrolase by the carbocyclic nucleoside 1.[17] The results of these experiments suggest that L-929 cells contain two forms of AdoHcy hydrolase: one form representing 90-95% of the total enzyme activity which is very sensitive to inactivation by 1; and a second form representing 5-10% of the total enzyme activity which is very insensitive to inactivation by 1. The nature and function of these two forms of AdoHcy hydrolase have yet to be determined.

Recently De Clercq[1] reported that combinations of AdoHcy hydrolase inhibitors and Hcy produced synergistic inhibition of viral replication. This observation stimulated us to conduct experiments to determine whether the inhibitor-insensitive form of AdoHcy hydrolase might be catalyzing the conversion of adenosine and Hcy to AdoHcy, thus providing a molecular

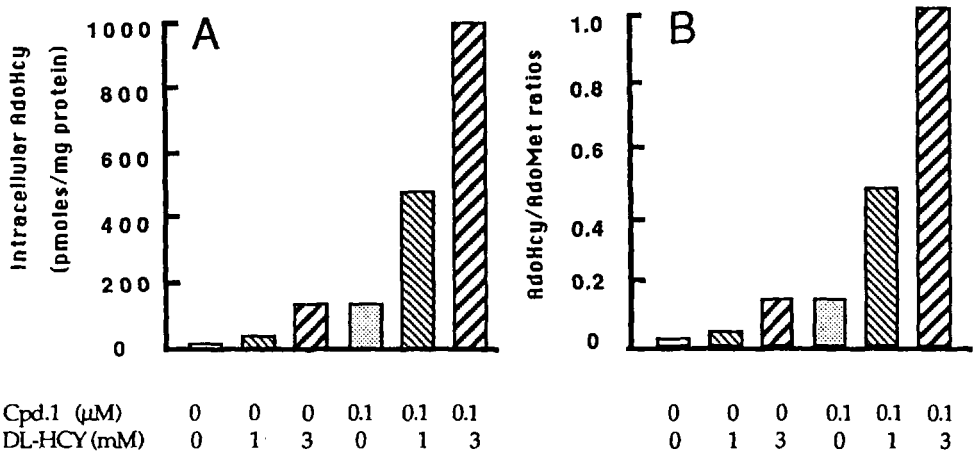


FIGURE 2. Effects of carbocyclic nucleoside 1 and Hcy alone or in combination on the accumulation of intracellular AdoHcy and AdoHcy/AdoMet ratios in L-929 cells. Monolayer cultures of L-929 cells in 6 cm dishes were incubated with 1 and Hcy alone or in combination for 24 hours. After harvest, the contents of AdoHcy and AdoMet from cell extracts were determined using HPLC analysis. A : Intracellular content of AdoHcy. B : AdoHcy/AdoMet ratios. Detail of methods for cell culturing, preparation of samples for HPLC and conditions for HPLC analysis of AdoHcy and AdoMet were reported previously.[7] Each result is the average of duplicate assays.

mechanism to explain the synergistic effects of AdoHcy hydrolase inhibitors and Hcy on inhibition of viral replication.

When L-929 cells were treated with carbocyclic nucleoside 1 and Hcy in various combinations for 24 hours, we observed synergistic increases in the intracellular levels of AdoHcy (Figure 2A). The resulting AdoHcy/AdoMet ratios for the drug combinations showed similar increases(Figure 2B). It should be noted that the intracellular levels of AdoMet are relatively constant (approx. 800-1000 pmoles/mg protein) regardless of the drug treatment. Similar to the earlier report by De Clercq[1], we have observed[18] synergistic effects of combinations of carbocyclic nucleoside 1 and Hcy on inhibition of vaccinia virus replication. The data described above suggest that the synergistic effects of combinations of 1 and Hcy on cellular AdoHcy is probably the molecular mechanism responsible for the synergistic effects on inhibition of viral replication.

In order to determine the biosynthetic source of AdoHcy in cells treated with carbocyclic nucleoside 1 and Hcy, we conducted double labelling experiments using [2,8-^{3}H]-adenosine ([^{3}H]-Ado) and [^{35}S]-methionine ([^{35}S]-Met). These double labelling experiments should allow for

TABLE 1. Relationship between cellular AdoHcy hydrolase activity and intracellular AdoHcy levels in L-929 cells treated with carbocyclic nucleoside 1 and Hcy alone or in combination*.

Hcy	compound 1: Experiment	0 μM AdoHcy	0 μM AdoMet	0.1 μM AdoHcy	0.1 μM AdoMet
0 mM	AdoHcy hydrolase activity (%)	100%		9%	
	[^{3}H]-activity (dpm)	8,535	108,091	17,358	121,486
	[^{35}S]-activity (dpm)	1,982	40,145	4,930	38,393
	Ratio of [^{3}H]/[^{35}S]	4.3	2.7	3.5	3.1
3 mM	AdoHcy hydrolase activity (%)	73%		17%	
	[^{3}H]-activity (dpm)	196,061	113,735	167,975	117,505
	[^{35}S]-activity (dpm)	0	37,361	0	38,794
	Ratio of [^{3}H]/[^{35}S]	∞	3.0	∞	3.0

*Duplicate sets of L-929 cell cultures were treated with compound 1 and Hcy alone or in combination. Confluent cultures in 6 cm dishes were incubated for one hour, pulse-labeled with [2,8-^{3}H]-Ado (7.2 μCi/mL) and [^{35}S]-Met (12.3 μCi/ml) for one hour, and harvested. Details of methods for the AdoHcy hydrolase assay, for cell culturing, preparation of samples for HPLC, and conditions for HPLC analysis of AdoMet and AdoHcy were reported previously[7]. The ratios of [^{3}H]/[^{35}S] in AdoHcy and AdoMet were determined after HPLC purification of these metabolites.

determination of the biosynthetic pathway responsible for the formation of AdoHcy since conversion of [^{35}S]-Met and [^{3}H]-Ado, the latter via ATP, to AdoHcy via AdoMet and transmethylation should produce [^{3}H]/[^{35}S]-AdoHcy having a [^{3}H]/[^{35}S] ratio equal to the [^{3}H]/[^{35}S] ratio of AdoMet. If AdoHcy is being produced by the AdoHcy hydrolase-catalyzed conversion of [^{3}H]-Ado and Hcy, the ratio of [^{3}H]/[^{35}S] in AdoHcy should be greater than the ratio in AdoMet.

Table 1 shows the results of experiments in which L-929 cells were treated with carbocyclic nucleoside 1 (0.1 μM) and Hcy (3 mM) alone or in combination for two hours, and pulse-labeled with [^{3}H]-Ado and [^{35}S]-Met during the second hour. In cells receiving no drug treatment and in cells treated with 1 alone, the [^{3}H]/[^{35}S] ratios in AdoHcy were 4.3 and 3.5, respectively. In contrast, in cells treated with Hcy alone or in cells treated with carbocyclic nucleoside 1 and Hcy in combination, the [^{3}H]/[^{35}S] ratios were infinity which resulted from an increased incorporation of [^{3}H]-Ado and a decreased incorporation of [^{35}S]-Met. In the same experiments, residual AdoHcy hydrolase activities in cells treated with compound 1 alone or in combination with Hcy were 9% and 17%, respectively. The [^{3}H]/[^{35}S] ratios in AdoMet were

not significantly affected by addition of either 1 or Hcy to the culture medium, increasing only slightly (ca. 10%) from the ratio observed in the control experiment. The amount of radioactivity ([^{3}H], [^{35}S]) in AdoMet (Table 1) and the absolute intracellular levels of AdoMet were both unaffected. This increased incorporation of [^{3}H]-Ado into AdoHcy shows that the formation of AdoHcy in cells treated with the combination of compound 1 and Hcy was catalyzed by the residual AdoHcy hydrolase activity.

In summary, we have shown in this study a synergistic accumulation of AdoHcy when murine L-929 cells are treated with a combination of Hcy and the AdoHcy hydrolase inhibitor 1. Under these experimental conditions, the increase in intracellular AdoHcy arises from the inhibitor-insensitive AdoHcy hydrolase catalyzing the conversion of Hcy and adenosine to AdoHcy. These results provide a molecular mechanism to explain the synergistic antiviral effects observed with combinations of AdoHcy hydrolase inhibitors and Hcy.[1,18] Of particular interest in our laboratory in the future will be the nature and physiological roles of the inhibitor-sensitive and inhibitor-insensitive forms of AdoHcy hydrolase.

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