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Adenosine Analogues as Substrates and Inhibitors of *S*-Adenosylhomocysteine Hydrolase in Intact Lymphocytes†

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ABSTRACT: A number of adenosine analogues have been examined for their ability to interact with *S*-adenosyl-L-homocysteine (SAH) hydrolase in intact mouse lymphocytes. In the presence of *erythro*-9-(2-hydroxy-3-nonyl)adenine, 3-deazaadenosine, 8-azaadenosine, formycin A, 2-aminoadenosine, 2-fluoroadenosine, N^6-methyladenosine, N^6-hydroxyadenosine, purine ribonucleoside and inosine were each metabolized to radioactive analogues of SAH when cells were labeled with either L-[2-^{3}H]methionine or L-[^{35}S]homocysteine. Tubercidin was shown to undergo metabolism both to *S*-[^{3}H]tubercidinyl-L-methionine and to *S*-[^{3}H]tubercidinyl-L-homocysteine in cells labeled with [2-^{3}H]methionine. 9-β-D-Arabinofuranosyladenine and 2′-deoxyadenosine caused marked elevations of [^{3}H]SAH in cells preloaded with [2-^{3}H]methionine but were not themselves metabolized detectably to SAH analogues. Adenine and 5′-deoxyadenosine also caused substantial elevations of [^{3}H]SAH under these same conditions. Some of the adenosine analogues shown to be metabolized to SAH analogues also caused an elevation of SAH in the lymphocytes. These results indicate the potential of adenosine analogues to interfere with cellular methylation reactions due either to their inhibition of SAH hydrolase or to their metabolism, via this enzyme, to SAH analogues.

Adenosine (Ado)[1] and many of its structural analogues are inhibitory to lymphocyte-mediated cytolysis (LMC) in vitro (Wolberg et al., 1975, 1978; Zimmerman et al., 1976). The LMC-inhibitory activity of the majority of these Ado analogues appears to be related to the ability of these agents to stimulate an elevation of adenosine 3′,5′-monophosphate (cAMP) within the cytolytic lymphocytes. However, a few compounds, such as 9-β-D-arabinofuranosyladenine (AraA), 2′-deoxyadenosine (2′-dAdo) and adenine, were found to be inhibitory to LMC in the absence of any appreciable effects on cAMP metabolism.

Several recent reports have drawn attention to the potential of Ado and its analogues to perturb cellular physiology due to their interaction with *S*-adenosyl-L-homocysteine (SAH) hydrolase (EC 3.3.1.1), the enzyme responsible for catabolism of SAH in eucaryotic cells (De La Haba & Cantoni, 1959; Walker & Duerre, 1975). The importance of this enzymatic reaction derives from the observations that SAH is a potent inhibitor of a number of *S*-adenosyl-L-methionine (SAM)-utilizing methyltransferases (Borchardt, 1977; Cantoni et al., 1979) and that cells appear unable to eliminate intact SAH across their plasma membrane (Walker & Duerre, 1975; Trewyn & Kerr, 1976). Since the equilibrium constant for SAH hydrolase favors greatly the synthesis of SAH over its hydrolysis (De La Haba & Cantoni, 1959), cells exposed simultaneously to Ado and L-homocysteine rapidly accumulate high levels of SAH (Kredich & Martin, 1977). Moreover, some Ado analogues, including 3-deazaadenosine (Chiang et al., 1977), AraA, 2′-dAdo, 5′-deoxyadenosine (5′-dAdo), and adenine (Hershfield & Kredich, 1978; Hershfield, 1979; Hershfield et al., 1979; Kredich et al., 1979; Palmers & Abeles, 1979; Ueland & Saebø, 1979), have been shown to be inhibitors of SAH hydrolase and, in the cases of 3-deazaadenosine and adenine, to cause an elevation of SAH within cells.

The recent finding that 3-deazaadenosine inhibits LMC and causes an elevation of lymphocyte SAH without any effect on cAMP levels indicates that SAH, like cAMP, is an intracellular modulator of this lymphocyte function (Zimmerman et al., 1978). It then became of interest to examine the interaction of other Ado analogues with SAH hydrolase as a possible basis for the inhibition of LMC by these agents. The present report describes the ability of a number of Ado analogues to serve as substrates or inhibitors of SAH hydrolase in mouse cytolytic lymphocytes.

Materials and Methods

L-[^{35}S]Homocysteine thiolactone (11 Ci/mol) and L-[2-^{3}H]methionine (2.9 Ci/mmol) were products of the Amersham Corp. *S*-[8-^{14}C]Adenosyl-L-homocysteine was kindly provided by Dr. E. M. Wise, Jr., and K. T. Calvert of these laboratories. *S*-3-Deazaadenosyl-L-homocysteine, *S*-8-azaadenosyl-L-homocysteine, *S*-[5′-(9-arabinofuranosyladenyl)]-L-homocysteine, *S*-tubercidinyl-L-homocysteine, and *S*-tubercidinyl-L-methionine were generous gifts from Dr. R. T. Borchardt of the University of Kansas (Borchardt et al., 1974, 1976a,b; Borchardt & Wu, 1975). SP-Sephadex C-25 was a product of Pharmacia Fine Chemicals. Boric acid gel was obtained from the Aldrich Chemical Co., Inc. The samples of adenine, 2′-dAdo, AraA, and 3′-deoxyadenosine (3′-dAdo) used in the present study were passed through small columns of boric acid

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[1] Abbreviations used: Ado, adenosine; AraA and adenine arabinoside, 9-β-D-arabinofuranosyladenine; cAMP, adenosine 3′,5′-monophosphate; 2′-dAdo, 2′-deoxyadenosine; 3′-dAdo, 3′-deoxyadenosine; 5′-dAdo, 5′-deoxyadenosine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; LC, high-performance liquid chromatography; LMC, lymphocyte-mediated cytolysis; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SAraAH, *S*-(5′-arabinofuranosyladenyl)-L-homocysteine; STH, *S*-tubercidinyl-L-homocysteine; STM, *S*-tubercidinyl-L-methionine.

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gel, under alkaline conditions, to eliminate possible contamination of these compounds by Ado (see below). Other materials were from sources identified elsewhere (Wolberg et al., 1978; Zimmerman et al., 1976, 1978).

Cytolytic lymphocytes were obtained from the peritoneal cavity of CD-1 mice after intraperitoneal injection and subsequent rejection of EL4 leukemia cells (Zimmerman et al., 1976). Dulbecco's phosphate-buffered saline supplemented with 10% fetal calf serum (heat inactivated) was used as the medium for the lymphocytes. *erythro*-9-(2-Hydroxy-3-nonyl)adenine (EHNA; 7.9 μM), an inhibitor of Ado deaminase (Schaeffer & Schwender, 1974), was included in all cellular incubations with Ado or its analogues, unless specified otherwise.

Metabolic Studies with L-[*2-*^{3}*H*]*Methionine.* Cytolytic lymphocytes [(1.2–3.7) $\times$ 10^8 cells in 10 mL of medium] were preincubated for 60 min at 37 °C with 100 μCi of L-[2-^{3}H]methionine and were then chilled and washed. The labeled cells were resuspended in fresh medium to a density of (1.6–3.3) $\times$ 10^6 cells/mL, and 5.0-mL portions of the cell suspensions were supplemented with saline or an Ado analogue and incubated for 30 min at 37 °C. Each experiment was performed in duplicate. Cells were harvested by centrifugation and were extracted with 5.0 mL of cold 1.0 M perchloric acid containing 2.0 μM 2-chloroadenosine as a recovery marker. The resultant extracts were clarified by centrifugation, neutralized with KOH, filtered through glass wool to remove the insoluble potassium perchlorate, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus), and reconstituted with 300 μL of 50 mM ammonium phosphate (pH 6.0). These samples were stored at −20 °C until their analysis by high-performance liquid chromatography.

Metabolic Studies with L-[^{35}*S*]*Homocysteine.* Cytolytic lymphocytes [(0.6–2.1) $\times$ 10^7 cells in 5.0 mL of medium] were supplemented with 200 μM L-[^{35}S]homocysteine plus either saline or an Ado analogue and were incubated for 30 min at 37 °C. Acid-soluble extracts of these lymphocytes were prepared as described above. In this case, however, each dried extract was reconstituted with 400 μL of deionized water.

Metabolic Studies with Tubercidin. Cytolytic lymphocytes (2.2×10^7 cells in 5.0 mL of medium) were supplemented with L-[2-^{3}H]methionine (0.7 μM) plus either saline or tubercidin (150 μM) and were incubated for 30, 60, or 90 min at 37 °C. EHNA was omitted from these cellular incubations. Cells were harvested by centrifugation and were extracted with 5.0 mL of cold 1.0 M perchloric acid containing 1.6 μM SAM and 0.8 μM SAH as recovery markers. Extracts were processed as above and reconstituted in 400 μL of deionized water. The excess of radioactive amino acids was removed from these extracts by loading the acidified samples onto small columns (0.55 $\times$ 5.0 cm) of SP-Sephadex, washing with 20 mL of 50 mM HCl, and eluting the *S*-nucleosidyl-L-amino acids with 10 mL of 500 mM HCl (Glazer & Peale, 1978). The column eluates were evaporated to dryness under reduced pressure and reconstituted with 250 μL of deionized water.

High-Performance Liquid Chromatography (LC) of Extracts. Acid-soluble extracts of cells were analyzed for radioactive metabolites of [2-^{3}H]methionine and of [^{35}S]-homocysteine by either reversed-phase LC (Zimmerman et al., 1978) and/or cation-exchange LC (Zimmerman et al., 1979). Fifty- or one-hundred-microliter samples of each cell extract were injected into the chromatograph. Full-scale absorbance (254 and 280 nm) ranges of 0.04 *A* unit were employed. The effluent of the chromatograph was collected at 1.0-min intervals, and these fractions were either monitored in a liquid scintillation spectrometer or pooled and desalted for further analysis. In reversed-phase LC, concentrations of 2-chloroadenosine present in cell extracts were calculated from a response factor (ultraviolet peak area per nanomole of 2-chloroadenosine) determined by injecting a known amount of 2-chloroadenosine into the chromatograph. The amount of 2-chloroadenosine present in each cell extract was used to normalize radioactivity measurements to the original cell count.

Other Techniques. Boric acid gel was washed first with 1.0 M and then with 0.1 M ammonium hydroxide. For use in purifying reagents, columns were packed with this gel to dimensions of 0.55 $\times$ 5.0 cm. 2′-dAdo, AraA, 3′-dAdo, or adenine (20–25 mg) was dissolved in 15 mL of 0.1 M ammonium hydroxide and passed through a column of boric acid gel. The eluate was evaporated to dryness under reduced pressure, and the recovered solid was dried further in a drying pistol with an ethanol reflux. For use with cell extracts, columns were packed with boric acid gel to dimensions of 0.55 $\times$ 2.0 cm. Samples (500 μL) were made 0.1 M in ammonium hydroxide, giving pH values of >9, and loaded onto the columns. Each column was washed with 1.0 mL of 0.1 M ammonium hydroxide, and the total eluates (1500 μL) were evaporated to dryness and reconstituted with 50 μL of deionized water.

Pooled fractions (4 mL) of radioactive metabolites which were collected after elution from reversed-phase LC were acidified and desalted on small columns of SP-Sephadex by the same methodology described above for removal of radioactive amino acids from cells extracts.

Results

Effect of Ado Analogues on Lymphocyte SAH Levels. The objective of the present study was to examine a variety of Ado analogues for both substrate and inhibitory activities toward SAH hydrolase in mouse cytolytic lymphocytes. In order to test compounds for inhibition of cellular SAH hydrolase, it is essential to have a highly sensitive assay capable of determining relative intracellular levels of SAH. Early in the course of this work it was recognized that the extremely small pool (<20 pmol/10^6 cells) of lymphocyte SAH, considered together with the paucity of tissue available for experimentation, rendered direct measurement of cellular SAH by LC ultraviolet flow monitors impractical. Therefore, it was decided to examine Ado analogues for their ability to inhibit SAH hydrolase by preloading lymphocytes with L-[2-^{3}H]methionine and by determining the effect of each Ado analogue on the flow of radioisotope along the pathway

L-methionine → SAM → SAH ⇄ Ado + L-homocysteine

Fractionation of acid-soluble extracts from control and drug-treated cells by reversed-phase LC allowed a determination of the relative amount of [2-^{3}H]methionine-derived radiolabel present in SAH under these different experimental conditions. In addition, this experimental approach has the advantage of simultaneously allowing detection of [^{3}H]SAH analogues formed metabolically from the different Ado analogues (see below).

Figure 1A illustrates the separation afforded by reversed-phase LC of authentic samples of SAM, SAH, *S*-[5′-(9-arabinofuranosyladenyl)]-L-homocysteine (SAraAH), and 2-chloroadenosine. Under these chromatographic conditions, [^{3}H]methionine was eluted in the breakthrough region (4–6 min) of the chromatogram. Extracts from [2-^{3}H]-methionine-preloaded control cells yielded only two prominent peaks of radioactivity, one corresponding to methionine and one coincident with SAM (Figure 1B). Control cells contained

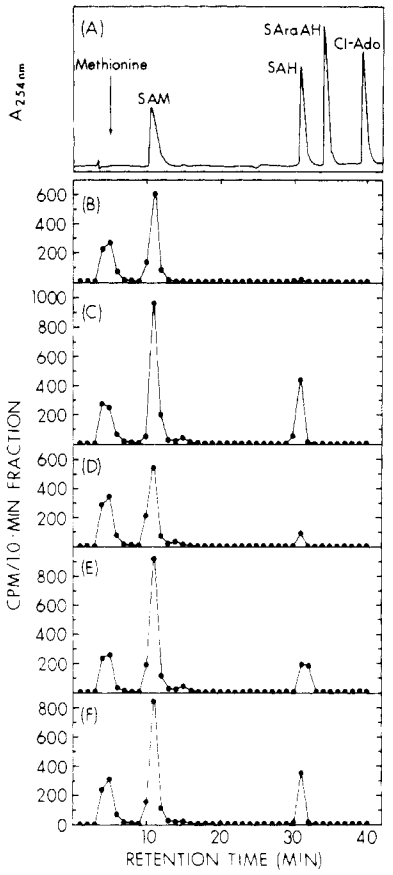

FIGURE 1: Reversed-phase LC of standards and extracts from drug-treated, [2-^{3}H]methionine-preloaded lymphocytes. Panel A shows the ultraviolet (254 nm) elution profile of several standard compounds: SAM, SAH, SAraAH, and 2-chloroadenosine (Cl-Ado). Radioactive methionine is eluted at 4–6 min in this chromatographic system. Panels B–F show the radioactive elution profiles of extracts from cells incubated for 30 min with (B) saline, (C) 150 μM AraA, (D) 150 μM 2′-dAdo, (E) 2.0 mM adenine, or (F) 10 μM Ado.

very little [^{3}H]SAH, usually <5% of the amount of [^{3}H]SAM. By contrast, extracts from [2-^{3}H]methionine-preloaded cells which had been incubated for 30 min with 150 μM AraA (Figure 1C), 150 μM 2′-dAdo (Figure 1D), 2.0 mM adenine (Figure 1E), or 10 μM Ado (Figure 1F) each yielded an enlarged peak of radioactivity at the retention time (31–32 min) for SAH.

Table I (third column) summarizes the ability of the various Ado analogues to cause a buildup of [^{3}H]SAH during a 30-min drug treatment of [2-^{3}H]methionine-preloaded lymphocytes. At the concentrations tested, Ado, 3-deazaadenosine, 5′-dAdo, 2′-dAdo, AraA, and adenine each caused an elevation of lymphocyte [^{3}H]SAH of fourfold or more above control values.[2] The somewhat varied concentrations of the different Ado analogues chosen for testing in Table I reflect the varying potencies of these agents as inhibitors of LMC (Wolberg et al., 1978).

The elevation of lymphocyte [^{3}H]SAH by AraA (Figure 2A), 2′-dAdo (Figure 2B), and adenine (Figure 2C) was dependent upon the concentration of each agent added to the [2-^{3}H]methionine-preloaded cells. AraA and 2′-dAdo caused maximal buildup of [^{3}H]SAH at intermediate concentrations

[2] A 4.0-fold elevation of [^{3}H]SAH was chosen as a cutoff for discussion purposes in view of the fact that 150 μM 8-azaadenosine caused a 3.4-fold buildup of [^{3}H]SAH even though this Ado analogue is not inhibitory to LMC under these same experimental conditions (Wolberg et al., 1978).

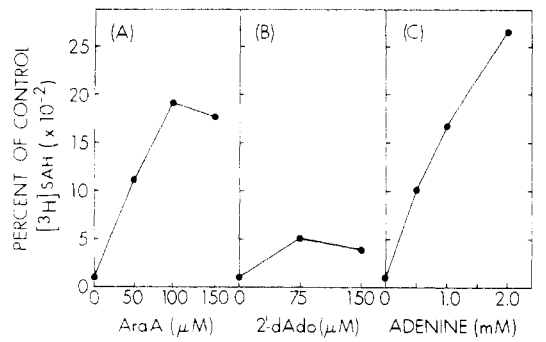

FIGURE 2: Concentration dependence of [^{3}H]SAH elevation caused by (A) AraA, (B) 2′-dAdo, and (C) adenine in [2-^{3}H]methionine-preloaded lymphocytes. Cells were incubated with each agent for 30 min prior to their acid extraction for subsequent analysis by reversed-phase LC. The experiment in panel C was conducted in the absence of EHNA.

and appeared to be less effective in this capacity at higher concentrations. The cellular buildup of [^{3}H]SAH caused by adenine (2.0 mM) was shown to reach its maximal value within 15 min after the addition of adenine to [2-^{3}H]-methionine-preloaded cells (results not shown). The rate of elimination of drug-elevated [^{3}H]SAH after transfer of the lymphocytes to drug-free medium varied among the different agents tested (Table II). Whereas intracellular levels of [^{3}H]SAH which were initially elevated by either adenine or 2′-dAdo fell rapidly after removal of these agents from the medium, the AraA-stimulated elevation of [^{3}H]SAH was less than 50% reduced after a 30-min incubation of the cells in drug-free medium.

Evidence for Metabolic Formation of SAH Analogues. Reversed-phase LC of extracts of [2-^{3}H]methionine-preloaded lymphocytes has provided evidence for the metabolism of several Ado analogues to their corresponding 5′-deoxy-5′-*S*-L-homocysteinyl derivatives. Extracts from cells which had been incubated for 30 min with 8-azaadenosine (Figure 3B), 3-deazaadenosine (Figure 3C), N^6-methyladenosine (Figure 3D), inosine (Figure 3E), 2-fluoroadenosine (Figure 3F), formycin A (Figure 3G), N^6-hydroxyadenosine (Figure 3H), or 2-aminoadenosine (Figure 3I) each yielded a unique radioactive metabolite of [2-^{3}H]methionine, with a retention time similar to that of SAH, which was absent in extracts from control cells (cf. Figure 1B). In the two cases where authentic SAH analogues were available as chromatographic standards, the novel radioactive metabolite peaks found in cells treated with 8-azaadenosine (Figure 3B) and 3-deazaadenosine (Figure 3C) were eluted with the same retention times (28–29 min) as *S*-(8-azaadenosyl)-L-homocysteine and *S*-(3-deazaadenosyl)-L-homocysteine (Figure 3A). In none of the above cases was sufficient material available to allow ultraviolet characterization of the putative SAH analogues. Table I (fourth column) summarizes which of the Ado analogues examined appeared to be metabolized to radioactive analogues of SAH in [2-^{3}H]methionine-preloaded cells. Under the conditions of the above experiment, 150 μM purine ribonucleoside appeared to cause a large elevation of [^{3}H]SAH; however, subsequent studies (see below) showed this result to be due to coelution of the corresponding SAH analogue with SAH from the reversed-phase column. Although *S*-tubercidinyl-L-homocysteine (STH) was not formed in detectable amounts from 150 μM tubercidin during the 30-min incubation period employed above, this SAH analogue was formed in cells after longer periods of tubercidin treatment (see below).

As an alternative way of investigating analogue SAH formation metabolically, lymphocytes were incubated for 30 min

Table I: Effect of Ado Analogues on the Metabolism of L-[2-^{3}H]Methionine and L-[^{35}S]Homocysteine in Mouse Cytolytic Lymphocytes[a]

compd	concn of compd tested (μM)	% of control [^{3}H]SAH value after 30-min incubn with compd	evidence for analogue SAH formation using as precursor	
			[2-^{3}H]Met	[^{35}S]Hcy
adenosine	10.0	2110	(+)[b]	(+)[b]
2-fluoroadenosine	4.7	73	+ (17)[c]	+
2-chloroadenosine	18.8	317	–	–
3-deazaadenosine	20.0	1310	+ (0.3)	+
N^6-benzyladenosine	150	120	–	–
N^6-phenyladenosine	150	385	–	–
5′-chloro-5′-deoxyadenosine	150	175	–	ND[d]
5′-deoxyadenosine	150	525	–	–
2′-*O*-methyladenosine	150	212	–	–
2′-deoxyadenosine	150	588	–	–
2-aminoadenosine	150	105	+ (20)	+
adenine arabinoside	150	2730	–	–
2-methyladenosine	150	191	–	–
2-methylthioadenosine	150	125	–	–
N^6-(Δ^2-isopentenyl)adenosine	150	*e*	–	–
N^6-methyladenosine	150	218	+ (1.8)	+
N^6-furfuryladenosine	150	*e*	–	ND
zeatin ribonucleoside	150	139	–	ND
3′-deoxyadenosine	150	159	–	–
3′-*O*-methyladenosine	150	123	–	–
formycin A	150	64	+ (28)	+
tubercidin	150	363	+[f]	–
N^6-hydroxyadenosine	150	*e*	+	+
purine ribonucleoside	150	105	+ (20)	+
6-methylmercaptopurine ribonucleoside	150	203	–	–
8-azaadenosine	150	335	+ (1.4)	+
8-bromoadenosine	150	279	–	–
inosine	150	108	+ (2.8)	+
adenine	2000	2650	–	–

[a] All compounds were tested for 30 min in the presence of 7.9 μM EHNA. Columns 4 and 5 denote whether radioactive SAH analogues were synthesized when mouse cytolytic lymphocytes were incubated for 30 min with the specified radioactive amino acid plus each Ado analogue. (+) means that the corresponding SAH analogue was formed and (–) means that no SAH analogue formation was detected. [b] These (+) symbols represent greatly elevated levels of radioactive SAH in the Ado-treated cells. [c] Values in parentheses give ratios of [^{3}H]SAH analogue/[^{3}H]SAH found in the extracts from [2-^{3}H]methionine-preloaded cells after 30-min incubation with each Ado analogue. [d] ND, not done. [e] Commerical samples of N^6-(Δ^2-isopentenyl)adenosine, N^6-furfuryladenosine, and N^6-hydroxyadenosine contained an Ado contamination which obscured the true effect of these compounds on lymphocyte levels of [^{3}H]SAH. [f] Evidence for [^{3}H]STH formation in lymphocytes derives from the experiment shown in Figure 5 and not from the 30-min drug incubations using [2-^{3}H]methionine-preloaded cells.

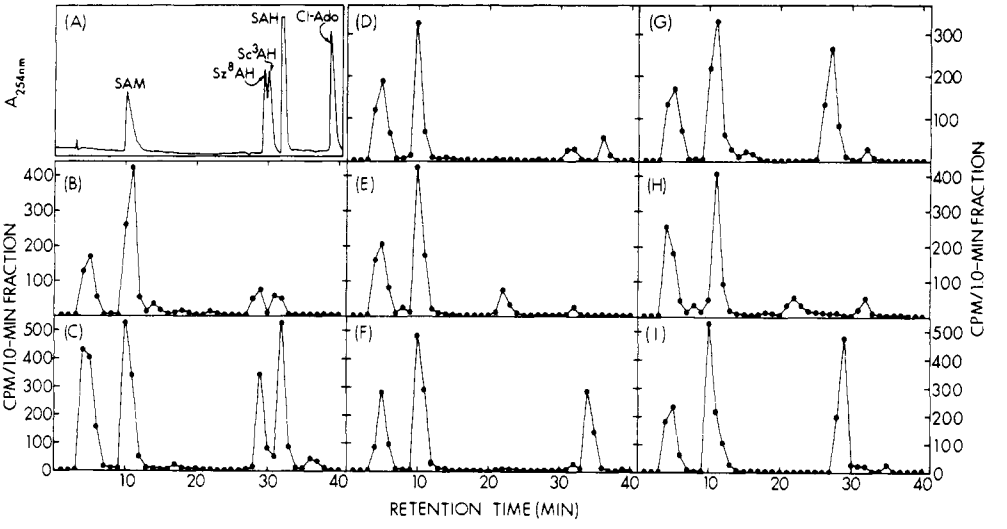

FIGURE 3: Reversed-phase LC of standards and extracts from drug-treated, [2-^{3}H]methionine-preloaded lymphocytes. Panel A shows the ultraviolet (254 nm) elution profile of several standard compounds: SAM, *S*-(8-azaadenosyl)-L-homocysteine (Sz^{8}AH), *S*-(3-deazaadenosyl)-L-homocysteine (Sc^{3}AH), SAH, and 2-chloroadenosine (Cl-Ado). Panels B–I show the radioactive elution profiles of extracts from cells incubated for 30 min with (B) 8-azaadenosine, (C) 3-deazaadenosine, (D) N^6-methyladenosine, (E) inosine, (F) 2-fluoroadenosine, (G) formycin A, (H) N^6-hydroxyadenosine, or (I) 2-aminoadenosine. All nucleosides except 3-deazaadenosine (5.0 μM) and 2-fluoroadenosine (4.7 μM) were present at 150 μM in these cellular incubations.

with 200 μM L-[^{35}S]homocysteine in the absence or presence of each of the different Ado analogues. As shown in Figure 4A, reversed-phase LC of an extract from control cells yielded two large peaks of radioactivity in the breakthrough region of the chromatogram and a small peak of radioactivity at the retention time (31–32 min) for SAH. Under these experimental conditions, N^6-methyladenosine (Figure 4B), formycin A (Figure 4D), 8-azaadenosine (Figure 4E), inosine (Figure

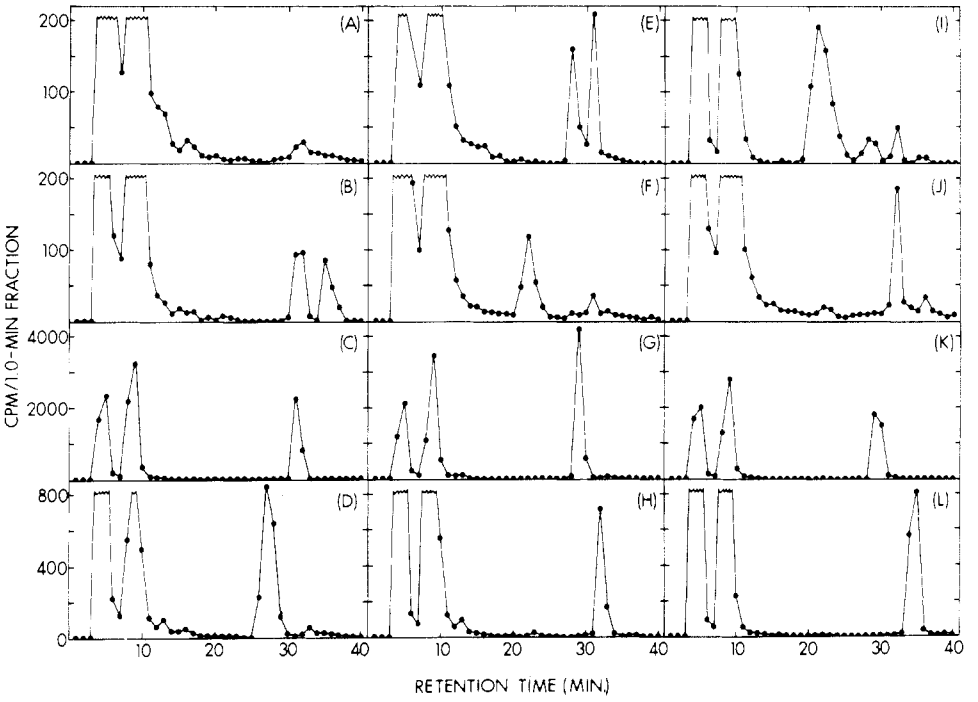

FIGURE 4: Reversed-phase LC of extracts from drug-treated, [^{35}S]homocysteine-labeled lymphocytes. Cells were incubated for 30 min with 200 μM [^{35}S]homocysteine plus (A) saline, (B) N^6-methyladenosine, (C) Ado, (D) formycin A, (E) 8-azaadenosine, (F) inosine, (G) 2-aminoadenosine, (H) purine ribonucleoside, (I) N^6-hydroxyadenosine, (J) tubercidin, (K) 3-deazaadenosine, or (L) 2-fluoroadenosine. All nucleosides except Ado (5.0 μM), 3-deazaadenosine (5.0 μM), and 2-fluoroadenosine (4.7 μM) were present at 150 μM in these cellular incubations. Note the different scale values of radioactivity used in the different rows of elution profiles.

Table II: Reversibility of Lymphocyte Elevation of [^{3}H]SAH Caused by Adenine, AraA, and 2′-dAdo[a]

treatment schedule	[^{3}H]SAH (dpm/10^6 cells)
saline (30 min)	39.4 ± 1.0
saline (30 min), wash cells, saline (30 min)	3.9 ± 2.2
saline (30 min plus 30 min)	24.4 ± 1.1
adenine (30 min)	773 ± 21
adenine (30 min), wash cells, saline (30 min)	24.8 ± 1.7
adenine (30 min plus 30 min)	457 ± 60
AraA (30 min)	998 ± 8
AraA (30 min), wash cells, saline (30 min)	599 ± 67
AraA (30 min plus 30 min)	1109 ± 107
2′-dAdo (30 min)	177 ± 26
2′-dAdo (30 min), wash cells, saline (30 min)	52.9 ± 2.7
2′-dAdo (30 min plus 30 min)	282 ± 11

[a] [2-^{3}H]Methionine-preloaded lymphocytes were incubated for 30 min at 37 °C in sextuplet with saline, adenine (2.0 mM), AraA (150 μM), or 2′-dAdo (150 μM), after which duplicate samples of cells from each experimental condition were (1) acid-extracted immediately, (2) washed twice, resuspended, and incubated for 30 min in drug-free medium, or (3) centrifuged, resuspended in the selfsame medium, and incubated for another 30 min prior to acid extraction. Cellular levels of [^{3}H]SAH were determined by reversed-phase LC.

4F), 2-aminoadenosine (Figure 4G), N^6-hydroxyadenosine (Figure 4I), 3-deazaadenosine (Figure 4K), and 2-fluoroadenosine (Figure 4L) each yielded an ^{35}S-labeled metabolite having a retention time close to, but distinct from, that of SAH. Ado (Figure 4C), purine ribonucleoside (Figure 4H), and tubercidin (Figure 4J) each gave rise to an enlarged peak of radioactivity having the same retention time as SAH. When extracts of [2-^{3}H]methionine-preloaded cells (cf. Figure 3) were mixed with corresponding extracts of [^{35}S]homocysteine-labeled cells and were then fractionated by reversed-phase LC, the ^{3}H- and ^{35}S-labeled metabolites formed from each Ado analogue were found to cochromatograph. Moreover, when extracts from [^{35}S]homocysteine-labeled cells were mixed with [^{14}C]SAH and subjected to reversed-phase LC, the putative ^{35}S-labeled SAH analogues formed from N^6-methyladenosine, formycin A, 8-azaadenosine, inosine, 2-aminoadenosine, N^6-hydroxyadenosine, 3-deazaadenosine, and 2-fluoroadenosine were each found to be eluted apart from the [^{14}C]SAH. It is noteworthy that no evidence was found for ^{35}S-labeled metabolites of any of the ribose-modified Ado analogues examined (AraA, 2′-dAdo, and 3′-dAdo). These results obtained with [^{35}S]homocysteine are summarized in Table I (fifth column).

Tubercidin and purine ribonucleoside required additional study in order to determine the effect of these two agents on lymphocyte levels of SAH and to discern whether these two Ado analogues are metabolized to corresponding SAH analogues. Each of these two agents caused an enlargement of the peak of radioactivity eluted from the reversed-phase column at the retention time of SAH, both in the experiment with [2-^{3}H]methionine-labeled cells (results not shown) and in the experiment with [^{35}S]homocysteine-labeled cells (Figure 4H,J). The availability of an authentic sample of STH allowed us to determine that STH is coeluted with SAH from the reversed-phase column. For this reason, cation-exchange LC was investigated and was found to yield good resolution of STH and SAH, as well as of *S*-tubercidinyl-L-methionine (STM) and SAM (Figure 5A). When the enlarged peaks of ^{3}H- or ^{35}S-labeled SAH present in extracts of tubercidin-treated cells were collected from the reversed-phase column, desalted on SP-Sephadex, and fractionated by cation-exchange LC, radioactivity was eluted only at the retention time of SAH

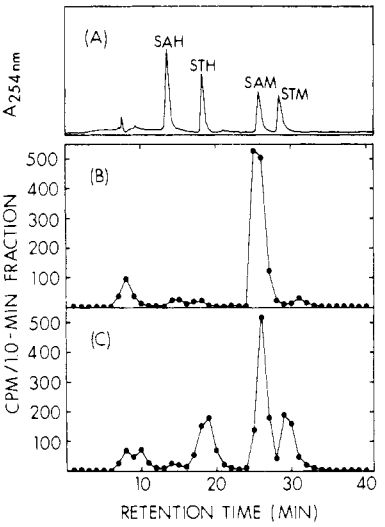

FIGURE 5: Cation-exchange LC of standards and extracts from control and tubercidin-treated, [2-^{3}H]methionine-labeled lymphocytes. Panel A shows the ultraviolet (254-nm) elution profile of several standard compounds. Panels B and C show the radioactive elution profiles of extracts from cells which had been incubated for 60 min with [2-^{3}H]methionine plus either (B) saline or (C) 150 μM tubercidin. This experiment was conducted in the absence of EHNA.

and not at the elution position of STH (results not shown). Thus, under the conditions of the above experiments (30-min incubations of cells with 150 μM tubercidin in the presence of 7.9 μM EHNA), it could be concluded that tubercidin caused an elevation of radioactive SAH in the lymphocytes and was not itself metabolized detectably to STH.

However, in view of the recent observation that STM formation is demonstrable during a more prolonged (2-h) incubation of cells with tubercidin (Zimmerman et al., 1979), STH formation was investigated during incubation times longer than 30 min. Accordingly, lymphocytes were incubated with L-[2-^{3}H]methionine for 30, 60, and 90 min, without EHNA, in the absence or presence of 150 μM tubercidin, and extracts from these cells were fractionated by cation-exchange LC. Cells incubated for only 30 min with tubercidin did not contain detectable amounts of either [^{3}H]STM or [^{3}H]STH. However, a 60-min incubation period was sufficient to allow metabolic formation of both [^{3}H]STM and [^{3}H]STH (Figure 5C). In this experiment it was notable that tubercidin did not cause an elevation of lymphocyte [^{3}H]SAH in the absence of EHNA (Figures 5B,C).

Cation-exchange LC has also proven useful in providing evidence that purine ribonucleoside is metabolized to its corresponding SAH analogue and that this Ado analogue does not cause an elevation in lymphocyte SAH. The enlarged peak of radioactivity eluted at the retention time of SAH with extracts from purine ribonucleoside treated cells, labeled either with [2-^{3}H]methionine (as in Figure 3) or with [^{35}S]homocysteine (Figure 4H), was collected after reversed-phase LC, desalted on SP-Sephadex, and then subjected to cation-exchange LC. As shown in parts B and D of Figure 6, most of the radioactivity derived from either [2-^{3}H]methionine or [^{35}S]homocysteine which was found in extracts from purine ribonucleoside treated cells at the retention time of SAH in reversed-phase LC was eluted from the cation-exchange column well ahead of SAH. The total separation of the [2-^{3}H]methionine-derived metabolite of purine ribonucleoside (shown in Figure 6B) from added [^{14}C]SAH (Figure 6C) provided confirmation of the uniqueness of this metabolite of purine ribonucleoside. In addition, this sequential combination of reversed-phase and cation-exchange LC has allowed us to

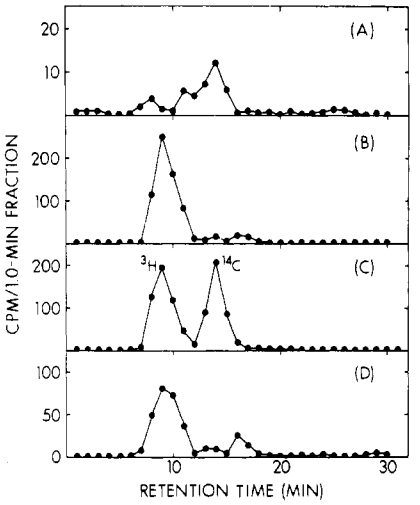

FIGURE 6: Cation-exchange LC of SAH fractions from lymphocyte extracts purified previously by reversed-phase LC. Panels A and B show the radioactivity elution profiles of SAH fractions from [2-^{3}H]methionine-preloaded cells which had been incubated for 30 min with either (A) saline or (B) 150 μM purine ribonucleoside. Panel C shows the elution profile obtained after mixing [^{14}C]SAH with a portion of the sample chromatographed in panel B. Panel D shows the radioactivity elution profile of the SAH fraction from cells which had been incubated for 30 min with 200 μM [^{35}S]homocysteine plus 150 μM purine ribonucleoside. Note the different scale values of radioactivity used for the different elution profiles.

determine that purine ribonucleoside treated cells (Figure 6B) did not contain elevated levels of [^{3}H]SAH relative to control cells (Figure 6A).

Lack of Metabolism of AraA and 2′-dAdo to SAH Analogues. In view of the wide variety of Ado analogues that were found to be metabolized to corresponding SAH analogues (Table I), additional experiments were performed to rule out the possibility that AraA and 2′-dAdo might also undergo a similar metabolism. [^{14}C]SAH was shown to cochromatograph in reversed-phase LC with the enlarged peaks of radioactivity presumed to be [^{3}H]SAH in extracts from AraA- (Figure 1C) and 2′-dAdo-treated (Figure 1D) cells, even though SAraAH is well separated from SAH in this chromatographic system (Figure 1A). Although authentic *S*-(2′-deoxyadenosyl)-L-homocysteine was not available as a chromatographic standard, this latter SAH analogue would be expected to be eluted after SAH based upon the observations that 2′-dAdo and dAMP are each retained longer than Ado and AMP, respectively, in this reversed-phase LC system.

Chromatography on boric acid gel also provided evidence that the radioactive material which accumulated in 2′-dAdo-treated cells was [^{3}H]SAH and not *S*-(2′-deoxyadenosyl)-L-[^{3}H]homocysteine. When a portion of the extract from 2′-dAdo-treated, [2-^{3}H]methionine-preloaded cells (Figure 1D) was supplemented with UV-measurable amounts of SAH and SAraAH and was then passed through a small column of boric acid gel under alkaline conditions, 65% of the added SAH (unlabeled), 62% of the endogenous [^{3}H]SAH, but none of the added SAraAH were removed from the sample.

Discussion

The present results demonstrate that a wide variety of Ado analogues can interact, as substrates and/or inhibitors, with SAH hydrolase in intact mouse lymphocytes. As such, this work both confirms and extends results, largely from enzymatic studies, reported recently. Formycin A, purine ribonucleoside (Guranowski & Pawelkiewicz, 1977), N^6-methyladenosine (Hoffman, 1978), and 3-deazaadenosine (Chiang et al., 1977)

have been reported to be substrates for SAH hydrolase from various sources, while N^6-methyladenosine and 3-deazaadenosine were also shown to undergo metabolism to corresponding SAH analogues in intact cells in these same studies. A number of Ado analogues, including 2′-dAdo, AraA, adenine, 5′-dAdo, and 3-deazaadenosine, have been reported to be inhibitors of SAH hydrolase isolated from several mammalian tissues (Chiang et al., 1977; Hershfield, 1979; Hershfield & Kredich, 1978; Hershfield et al., 1979; Kredich et al., 1979; Palmer & Abeles, 1979; Ueland & Saebø, 1979). To date, 3-deazaadenosine (Chiang et al., 1977) and adenine (Kredich et al., 1979) are the only Ado-like compounds which have been shown to cause an elevation of SAH in intact cells.

The substrate and inhibitory activities of the different Ado analogues observed in the present study suggest that ribose-modified derivatives (as exemplified by 2′-dAdo, AraA, adenine, and 5′-dAdo) may function chiefly as inhibitors of SAH hydrolase, while various base-modified derivatives can function either as substrates alone (e.g., 2-fluoroadenosine, 2-aminoadenosine, and formycin A) or as both substrates and inhibitors (e.g., 3-deazaadenosine) of this enzyme. The lymphocyte metabolism of the various Ado analogues to their corresponding SAH analogues was highly dependent upon the nature of the structural modification introduced into the purine moiety of Ado (Table I). Substrate activity with SAH hydrolase was retained partially when the 6-amino group of Ado was replaced by either a hydrogen atom or a hydroxyl group; however, 6-methylmercaptopurine ribonucleoside did not appear to be condensed with L-homocysteine intracellularly. Small substituents, such as methyl or hydroxyl moieties, were tolerated at the N^6 position; however, bulkier N^6 substituents, such as phenyl, benzyl, or isopentenyl groups, appeared to abolish substrate activity with SAH hydrolase. Among 2-substituted derivatives of Ado examined, 2-fluoro- and 2-aminoadenosine were metabolized extensively to their corresponding SAH analogues, while 2-chloro-, 2-methyl-, and 2-methylthioadenosine appeared to be unreactive with SAH hydrolase. 3-Deazaadenosine, the only structural modification in the pyrimidine ring of Ado examined, was metabolized efficiently to *S*-(3-deazaadenosyl)-L-homocysteine in the presence of abundant homocysteine (Figure 4K) but seemed to function chiefly as an inhibitor of SAH hydrolase under conditions of limiting homocysteine (Figure 3C). Modifications at the 7 and 8 positions in the imidazole ring of Ado gave strikingly different results: replacement of the 7-nitrogen by carbon, as in tubercidin, appeared to abolish substrate activity with SAH hydrolase; by contrast, replacement of the 8-carbon by nitrogen, as in 8-azaadenosine and formycin A, preserved substrate activity. 8-Bromoadenosine was not metabolized detectably to 8-BrSAH.

Tubercidin may be unique among the Ado analogues investigated in this study in that this nucleoside appears to have undergone metabolism to STH independently of SAH hydrolase. [^{35}S]STH was not formed in detectable amounts in lymphocytes incubated with [^{35}S]homocysteine plus tubercidin, under conditions where other Ado analogues were metabolized to their corresponding [^{35}S]SAH analogues. Tubercidin has previously been shown to be metabolized both to tubercidin 5′-triphosphate (Wolberg et al., 1978) and to STM (Zimmerman et al., 1979) in these cells. Lymphocytes labeled with [2-^{3}H]methionine converted tubercidin both to [^{3}H]STH and to [^{3}H]STM with similar time dependencies: while neither of these radioactive metabolites was detectable after a 30-min incubation of cells with 150 μM tubercidin, both metabolites were present in approximately equal amounts after a 60-min incubation (Figure 5). The above results are consistent with the finding that chemically synthesized STH is not a substrate for SAH hydrolase (Crooks et al., 1979) and suggest that STH is formed metabolically via one or more transmethylation reactions utilizing STM as the methyl donor. To the extent that this interpretation is valid, STH which is formed metabolically from STM would be expected to accumulate intracellularly and to cause progressivcly greater inhibition of STH-sensitive methylation reactions.

[2-^{3}H]Methionine-preloaded lymphocytes which were treated with AraA to bring about an elevation of [^{3}H]SAH and were then washed and transferred to drug-free medium appeared to eliminate the accumulated [^{3}H]SAH less rapidly than cells similarly treated with either 2′-dAdo or adenine (Table II). These results are consistent with the finding that, while both AraA and 2′-dAdo are irreversible inhibitors of SAH hydrolase, AraA is the more potent inhibitor (Hershfield, 1979). The decline in cellular [^{3}H]SAH observed in the continued presence of adenine (Table II) is interpreted to represent incomplete inhibition of SAH hydrolase by this agent.

3′-dAdo was much less effective than 2′-dAdo, 5′-dAdo, and AraA in causing an elevation of [^{3}H]SAH in [2-^{3}H]-methionine-preloaded lymphocytes (Table I). Recently, a preliminary report (Hershfield, 1979) has stated that 3′-dAdo, like 2′-dAdo and AraA, causes inactivation of human placenta SAH hydrolase *in the absence of substrates* (i.e., Ado or SAH). The relatively small effect of 3′-dAdo on levels of [^{3}H]SAH in mouse lymphocytes may indicate a differing inhibitory potency of 3′-dAdo toward SAH hydrolase from different species or tissues. Alternatively, sufficient Ado or SAH may be present in the lymphocytes to protect SAH hydrolase from inactivation by 3′-dAdo. Although Palmer & Abeles (1979) did not detect any inhibition of beef liver SAH hydrolase by 3′-dAdo or 2′-dAdo, the high concentration (0.4 mM) of [^{3}H]Ado employed by these authors in their assay for SAH hydrolase is sufficient to prevent inactivation of this enzyme by the Ado analogues (Hershfield, 1979).

SAH is known to be a potent inhibitor of a considerable number of SAM-utilizing methyltransferases (Borchardt, 1977; Cantoni et al., 1979), and agents, such as AraA, which are inhibitory to SAH hydrolase and cause an elevation of cellular SAH would be expected to interfere with physiological processes which are dependent upon SAH-sensitive methylation reactions. Indeed, a recent study has provided evidence that inhibition of LMC by 3-deazaadenosine is due proximally to the elevation of lymphocyte SAH caused by this Ado analogue (Zimmerman et al., 1978), and it is plausible that agents such as AraA, 2′-dAdo, 5′-dAdo, and adenine also inhibit LMC (Wolberg et al., 1978) via this same mechanism.

Obviously, the metabolic formation of nucleoside-modified analogues of SAH can have biological significance only if these agents affect some crucial metabolic process, presumably a methyltransferase. Six of the SAH analogues shown to be formed metabolically in mouse lymphocytes, *S*-(2-fluoroadenosyl)-L-homocysteine (Coward & Slisz, 1973; Coward et al., 1974), *S*-(3-deazaadenosyl)-L-homocysteine (Borchardt, 1975; Pugh et al., 1977; Leboy et al., 1978; Borchardt & Pugh, 1979), *S*-(N^6-methyladenosyl)-L-homocysteine (Borchardt et al., 1976a; Trewyn & Kerr, 1976; Pugh et al., 1977; Leboy et al., 1978; Borchardt & Pugh, 1979), STH (Coward et al., 1974; Borchardt et al., 1976a; Pugh et al., 1977; Leboy et al., 1978; Borchardt & Pugh, 1979), *S*-(8-azaadenosyl)-L-homocysteine (Borchardt et al., 1976a; Pugh et al., 1977; Leboy et al., 1978; Borchardt & Pugh, 1979), and *S*-inosyl-L-homo-

cysteine (Coward et al., 1974; Borchardt et al., 1974; Pugh et al., 1977), have been synthesized chemically and shown to exhibit varying inhibitory activities with several different methyltransferases. The possible significance of some of these SAH analogues in the cytolytic lymphocytes is indicated by the finding that homocysteine potentiates the LMC-inhibitory activity of 3-deazaadenosine (Zimmerman et al., 1978), 2-fluoroadenosine, 2-aminoadenosine, N^6-methyladenosine, formycin A, and 8-azaadenosine (G. Wolberg and G. S. Duncan, unpublished observations). Experiments are in progress attempting to evaluate the relevance of the present biochemical findings to the mechanism(s) by which the various Ado analogues inhibit LMC.

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