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Effects of 4′-Modified Analogs of Aristeromycin on the Metabolism of *S*-Adenosyl-L-homocysteine in Murine L929 Cells

DANA B. AULT-RICHÉ, YOUNHA LEE, CHONG-SHENG YUAN, MASAHIDE HASOBE, MICHAEL S. WOLFE, DAVID R. BORCHERDING, and RONALD T. BORCHARDT

Departments of Biochemistry (D.B.A.R., Y.L., C-S.Y., M.H., R.T.B.) and Medicinal Chemistry (M.S.W., D.R.B., R.T.B.), The University of Kansas, Lawrence, Kansas 66045

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SUMMARY

(1′*R*,2′*S*,3′*R*)-9-(2′,3′-Dihydroxycyclopentan-1′-yl)adenine (DHCaA), (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)-3-deazaadenine (3-deaza-DHCaA), (4′*R*)-4′-methyl-DHCaA, and (4′*R*)-4′-vinyl-DHCaA, which are analogs of the carbocyclic nucleoside aristeromycin, were synthesized earlier by our laboratory and were shown to be potent inhibitors of purified bovine liver *S*-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1). In the present study, these analogs were shown to produce rapid (within 15 min) and concentration-dependent (0.03–10 μM) inhibition of AdoHcy hydrolase in cultured murine L929 cells [relative order of inhibitory activity, DHCaA = 3-deaza-DHCaA ≫ (4′*R*)-4′-vinyl-DHCaA = (4′*R*)-4′-methyl-DHCaA]. The relative potencies of these inhibitors on the L929 AdoHcy hydrolase were consistent with their inhibitory effects on the recombinant forms of rat liver and human placental enzymes. This inhibition of L929 cellular AdoHcy hydrolase persisted for up to 48 hr. The inhibition of the L929 AdoHcy hydrolase resulted in a significant increase in the cellular concentrations of AdoHcy, whereas the cellular *S*-adenosylmethionine (AdoMet) levels remained relatively constant, thereby elevating the AdoHcy/AdoMet ratios. Maximum increases in AdoHcy levels and AdoHcy/AdoMet ratios occurred within 6 hr of exposure to the inhibitors and persisted for at least 24 hr. At a concentration of 1 μM, DHCaA and 3-deaza-DHCaA increased AdoHcy/AdoMet ratios to approximately 0.8 (after 24 hr of exposure to the inhibitors), whereas (4′*R*)-4′-vinyl-DHCaA and (4′*R*)-4′-methyl-DHCaA elevated AdoHcy/AdoMet ratios to approximately 0.15, compared with control levels of 0.05. Treatment of L929 cells with concentrations of DHCaA, 3-deaza-DHCaA, (4′*R*)-4′-vinyl-DHCaA, and (4′*R*)-4′-methyl-DHCaA up to 10 μM did not result in changes in cellular levels of endogenous nucleotides (e.g., CTP, UTP, ATP, and GTP). In contrast, cells treated with 10 μM aristeromycin for 6 hr contained reduced cellular levels of CTP, ATP, and GTP and significant levels of aristeromycin triphosphate and a GTP metabolite of this carbocyclic nucleoside. These data clearly show that the 4′-modified analogs [DHCaA, 3-deaza-DHCaA, (4′*R*)-4′-vinyl-DHCaA, and (4′*R*)-4′-methyl-DHCaA] retain inhibitory activity toward cellular AdoHcy hydrolase, causing elevated levels of AdoHcy and elevated AdoHcy/AdoMet ratios. However, these analogs are devoid of substrate or inhibitory activity toward cellular adenosine kinase. In addition, aristeromycin is rapidly metabolized in murine L929 cell lysates, i.e., >60% of the aristeromycin had been metabolized in 6 hr. In contrast, neither DHCaA nor 3-deaza-DHCaA showed any decrease in concentration after incubation with cell lysates for up to 6 hr.

AdoHcy hydrolase is a ubiquitous eukaryotic enzyme whose presumed physiological function is to catalyze the degradation of AdoHcy to Ado and Hcy, thus maintaining low cellular concentrations of this product inhibitor of AdoMet-dependent transmethylation reactions (Fig. 1) (1, 2). Inhibition of cellular AdoHcy hydrolase results in an intracellular accumulation of AdoHcy, causing a significant increase in the intracellular AdoHcy/AdoMet ratio and the subsequent inhibition of AdoMet-dependent methylations (3–8).

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Recently, AdoHcy hydrolase has become an attractive target for the design of broad-spectrum antiviral agents (8–11). The interest in AdoHcy hydrolase as a target for the design of antiviral agents has arisen because (a) most plant and animal viruses require a "capped methylated structure" at the 5′ terminus of their mRNA for viral transcription and thus viral replication (12), (b) a close correlation exists between the antiviral potencies of Ado analogs and their selective inhibitory effects on AdoHcy hydrolase (13), and (c) AdoHcy hydrolase inhibitors have been shown to be broad-spectrum antiviral agents possessing activity against poxviruses, (−)-stranded RNA viruses, and double-stranded RNA viruses (9) (see Ref.

ABBREVIATIONS: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; DHCaA, (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)adenine; 3-deaza-DHCaA, (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)-3-deazaadenine; Ado, adenosine; Hcy, homocysteine; DHCeA, (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopent-4′-enyl)adenine; HPLC, high performance liquid chromatography; PBS(−), phosphate-buffered saline lacking calcium ions; (4′*R*)-4′-methyl-DHCaA, (1′*R*,2′S,3′*R*,4′*R*)-9-(2′,3′-dihydroxy-4′-methylcyclopentanyl)adenine; (4′*R*)-4′-vinyl-DHCaA, (1′*R*,2′S,3′*R*,4′*R*)-9-(2′,3′-dihydroxy-4′-vinylcyclopentanyl)adenine.

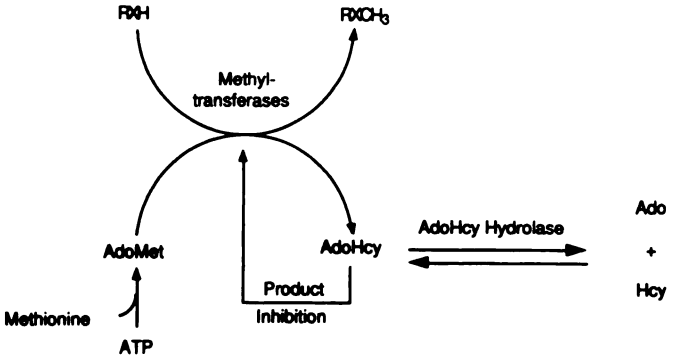
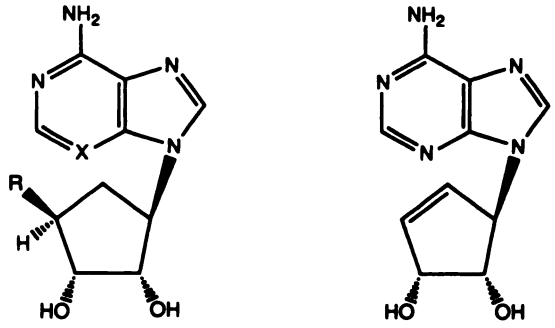


Fig. 1. Role of AdoHcy hydrolase in regulating AdoMet-dependent transmethylation reactions.



	X	R	
DHCaA	N	H	DHCeA
3-deaza DHCaA	CH	H	
(4'R)-4'-vinyl DHCaA	N	$CH{=}CH_2$	
(4'R)-4'-methyl DHCaA	N	CH_3	

Fig. 2. Structures of 4′-modified analogs of aristeromycin and DHCeA.

10 for a comprehensive review of the rationale for selecting AdoHcy hydrolase as a target for antiviral chemotherapy).

The first generation of AdoHcy hydrolase inhibitors included carbocyclic nucleosides such as aristeromycin (14, 15) and neplanocin A (4, 16). These naturally occurring carbocyclic nucleosides were shown to be very potent inhibitors of AdoHcy hydrolase ($K_i < 10$ nM); unfortunately, the cytotoxicity of these compounds precluded their clinical use as antiviral agents. By conducting metabolic, cytotoxicity, and antiviral activity studies on neplanocin A and synthetic analogs (see Ref. 10 for a comprehensive review), it has been clearly established that a significant part of the cytotoxicity of neplanocin A can be attributed to its ability to serve as a substrate for Ado kinase. In contrast, its antiviral effects have been shown to result from its ability to inhibit AdoHcy hydrolase. Based on these studies, highly specific and potent inhibitors of AdoHcy hydrolase have been designed and synthesized (17–20) and have been shown to be less cytotoxic than neplanocin A (21), while still retaining antiviral activity (22).

Another naturally occurring carbocyclic nucleoside known to inhibit AdoHcy hydrolase and exhibit antiviral activity is aristeromycin (14, 15). Like neplanocin A, aristeromycin is cytotoxic (15, 23), and this cytotoxicity may be related in part to its phosphorylation by kinases (24) or its conversion to a carbocyclic GMP analog (23). In an attempt to design analogs of aristeromycin that retain their inhibitory effects on AdoHcy hydrolase and exhibit antiviral activity with reduced cytotoxicity, our laboratory recently reported the synthesis of a series of 4′-modified analogs of aristeromycin (Fig. 2) (17). In this study we describe the effects of the most potent 4′-modified aristeromycin analogs [DHCaA, 3-deaza-DHCaA, (4′*R*)-4′-vinyl-DHCaA, and (4′*R*)-4′-methyl-DHCaA] on murine L929 cell AdoHcy hydrolase and cellular metabolism of AdoHcy, AdoMet, and endogenous nucleotides. The neplanocin A analog DHCeA was shown earlier by our laboratory to be a potent inhibitor of AdoHcy hydrolase and an effective antiviral agent with reduced cytotoxicty (18, 21). DHCeA was therefore used as a "benchmark" compound against which the 4′-modified analogs of aristeromycin could be compared.

Experimental Procedures

Materials. Standard chemicals and supplies were purchased from the following commercial suppliers: calf intestinal Ado deaminase, SP-Sephadex C-25, tri-*n*-octylamine, ammonium sulfate, egg white lysozyme, and DL-Hcy, Sigma Chemical Co. (St. Louis, MO); 3a70 scintillation cocktail, Research Products International (Mount Prospect, IL); Waymouth MB 752/1 (low calcium) medium for spinner culture, Waymouth MB 752/1 medium for dish culture, and calf bovine serum, JRH Biosciences (Lenexa, KS); sodium phosphate monobasic (HPLC grade), 1-heptanesulfonic acid, sodium salt (HPLC grade), and acetonitrile (HPLC grade), Fischer Scientific (Springfield, NJ); [2,8-^{3}H]Ado (36 Ci/mmol), Amersham (Arlington Heights, IL); Partisil-10 SAX HPLC column and Econosphere C-18 reverse phase HPLC column, Alltech (Deerfield, IL); Freon (1,1,2-trichlorotriflouroethane), Aldrich Chemical Co. (Milwaukee, WI); DEAE-Sepharose, Sephacryl-S300, and DEAE-cellulose, Pharmacia (Piscataway, NJ); ampicillin, Boehringer Mannheim (Indianapolis, IN); and isopropyl-β-D-thiogalactopyranoside, United States Biochemicals (Cleveland, OH).

[2,8-^{3}H]AdoHcy (7.1 mCi/mmol) was synthesized enzymatically from [2,8-^{3}H]Ado and DL-Hcy, using purified recombinant human placental AdoHcy hydrolase, according to the method of Chabannes *et al.* (25). Protein concentrations were determined according to the method of Bradford (26). DHCeA was synthesized according to procedures described earlier by our laboratory (19). DHCaA, (4′*R*)-4′-vinyl-DHCaA, and (4′*R*)-4′-methyl-DHCaA were synthesized by our laboratory according to published procedures (17). Aristeromycin was also synthesized in our laboratory according to published procedures (27).

Synthesis of 3-deaza-DHCaA. To a solution of (1′*R*,4′*R*,5′*S*)-9-(4′,5′-dihydroxy-2-cyclopenten-1-yl)adenine hydrochloride (0.42 mmol) (20) in methanol (100 ml) was added 0.044 mmol of platinum oxide. The suspension was then placed under hydrogen (25 psi) for 6 hr. Filtration through Celite and concentration afforded a quantitative yield of 3-deaza-DHCaA·HCl [m.p., 125°; $[\alpha]_D^{23} = -210°$ (c = 1.1, H_2O); ^{1}H NMR (80 MHz, dimethylsulfoxide-d_6 plus D_2O): 7.98 (s, 1 H), 7.58 (d, J = 8 Hz, 1 H), 6.46 (d, J = 8 Hz, 1 H), 4.42 (m, 1 H), 4.28 (dd, J = 9 and 4, 1 H), 3.95 (m, 1 H), and 2.20–1.55 (m, 4 H); mass spectrometry (electron impact): m/z 234 (M^+), 161, and 135].

Purification of recombinant human placental and rat liver AdoHcy hydrolases. Recombinant human placental and rat liver AdoHcy hydrolases were purified by a modified procedure described by Gomi *et al.* (28). *Escherichia coli* carrying the expression vector for either recombinant human placental (pPROKcd20) or rat liver (pPUCSAH) AdoHcy hydrolase were grown at 37° in 500 ml of 2× yeast/tryptone medium containing 17.5 mg of ampicillin. When the cell turbidity measured at 600 nm reached an absorbance of 0.2, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 2 mM for *E. coli* transformed with pPROKcd20 or 1 mM for *E. coli* transformed with pPUCSAH, and the culture was continued for an additional 14 hr. Cells were harvested by centrifugation, suspended in

20 ml of 50 mM Tris·HCl, pH 7.5, containing 2 mM EDTA, and lysed by treatment with egg white lysozyme (1 mg/ml) for 30 min at 0°, followed by brief sonication. A cell-free extract was prepared by centrifugation of the mixture at 105,000 × *g* for 1 hr at 4°. All additional purification steps were carried out at 4°. To remove nucleic acids, the cell-free extract from 1 liter of culture was mixed with DEAE-cellulose (DE52 Whatman) and equilibrated with 0.1 M potassium phosphate, pH 7.2, containing 1 mM EDTA. After mixing, the DEAE-cellulose was removed by filtration through a sintered glass funnel and the DEAE-cellulose was washed with an equal volume of 0.1 M potassium phosphate, pH 7.2, containing 1 mM EDTA. Ammonium sulfate was gradually added to 65% saturation and the solution was kept at 4° for at least 14 hr. The protein precipitate was collected by centrifugation at 13,000 × *g* for 30 min and the pellet was resuspended in 10 ml of buffer A (10 mM potassium phosphate, pH 7.2, containing 1 mM EDTA) and subjected to gel filtration on a column (2.8 × 105 cm) of Sephacryl S-300 that had been equilibrated and was eluted with 50 mM potassium phosphate, pH 7.2, containing 1 mM EDTA. Fractions with AdoHcy hydrolase activity were combined and concentrated, using a protein concentrator (Amicon) with a PM 30 ultrafiltration membrane, to a volume of 10 ml. The final purification was achieved by DEAE-Sepharose column chromatography (2.8 × 24 cm) with a linear gradient from 10 to 100 mM potassium phosphate, pH 7.2, containing 1 mM EDTA. Fractions containing AdoHcy hydrolase activity were combined, concentrated to approximately 1 mg/ml, and dialyzed against buffer A. After dialysis, samples were lyophilized and stored at −80°.

We were able to obtain consistent recoveries of 60 mg of human placental AdoHcy hydrolase from 1-liter cultures of *E. coli* transformed with pPROKcd20 (specific activity, 1.1 unit/mg) and 15 mg of rat liver AdoHcy hydrolase from 1-liter cultures of *E. coli* transformed with pPUCSAH (specific activity, 0.8 unit/mg). Both purified enzymes migrated as a single band (M_r 45,000) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Murine L929 cell cultures. Stocks of L929 cells were maintained continuously in suspension culture with Waymouth 752/1 medium, containing 4% calf serum, at 37°. For the experiments, L929 cells were grown as monolayers in Waymouth MB 752/1 medium containing 2% calf serum.

Determination of AdoHcy hydrolase inhibition constants. For the determination of inhibition constants, AdoHcy hydrolase was preincubated with various concentrations of inhibitors for varying times and the remaining enzyme activity was measured (as described in Ref. 17). The enzyme activity was determined by incubating 20 nM AdoHcy hydrolase with 0.2 mM Ado and 5 mM Hcy for 5 min at 37° in 50 mM potassium phosphate, pH 7.2, containing 1 mM EDTA, and assaying by HPLC the AdoHcy produced, after the reaction was stopped by addition of perchloric acid (final concentration, 0.5 N). An aliquot of 100 μl of the supernatant obtained after centrifugation of the reaction mixture was injected onto an HPLC column (Econosphere C-18 reverse phase column, 25 cm × 4.6 mm) and analyzed with a two-step gradient program (solvent A, acetonitrile; solvent B, 50 mM sodium phosphate, pH 3.2, containing 10 mM heptanesulfonic acid; program, 5–20% A for 15 min and 20–25% A for 10 min), at a flow rate of 1.0 ml/min. The peak area of AdoHcy was monitored at 254 nm to quantitate the amount of AdoHcy. The pseudo-first-order rate of inactivation (k_{obs}) was determined from a plot of the remaining activity versus preincubation time. K_i and k_2 were obtained from a plot of $1/k_{obs}$ versus 1/[inhibitor], using the equation $1/k_{obs} = K_i/(k_2[I]) + 1/k_2$.

Determination of cellular AdoHcy hydrolase activity. L929 cell monolayers (1×10^7 cells/10-cm dish) were treated with various amounts of inhibitors for various times, washed with cold PBS(−), and then harvested into microcentrifuge tubes. After centrifugation, the cell pellets were lysed in 400 μl of cold hypotonic buffer (10 mM Na_2HPO_4, 10 mM NaCl, 1.5 mM $MgAc_2$) by rapid freezing in dry ice/acetone and thawing. After centrifugation at 14,000 rpm for 2 min, the supernatant (320 μl) was mixed with a reaction mixture (final concentration, 150 mM potassium phosphate, pH 7.6, containing 1 mM EDTA, 40 μM [2,8-^{3}H]AdoHcy (7.1 mCi/mmol), and 4 units of Ado deaminase) and incubated for 30 min at 37°, as described previously by our laboratory (18). The reaction was stopped by the addition of 100 μl of 5 N formic acid. The reaction samples were then applied to columns (1.2 × 4 cm) of SP-Sephadex C-25 that had been equilibrated with 0.1 N formic acid. After prewashing with 2 ml of 0.1 N formic acid, [2,8-^{3}H]inosine was eluted with an additional 10 ml of 0.1 N formic acid. Radioactivity in 1 ml of the eluent sample was determined by liquid scintillation counting.

Determination of AdoHcy content and AdoHcy/AdoMet ratio. Intracellular AdoHcy and AdoMet concentrations were determined as reported previously by Hasobe *et al.* (29). L929 cell monolayers (3×10^6 cells/60-mm dish) were washed with cold PBS(−) and harvested into microcentrifuge tubes. After centrifugation, the cell pellets were lysed in 100 μl of 0.25 N perchloric acid by vortexing and rapid freezing and thawing. One hundred microliters of the supernatant obtained after centrifugation were analyzed by HPLC as described previously. The acid-insoluable pellets were dissolved in 0.1 N NaOH containing 0.2% Na_2CO_3, and the protein contents were measured according to the method of Bradford (26). AdoHcy/AdoMet ratios were calculated from the intracellular contents of AdoHcy and AdoMet.

Separation and quantification of ribonucleotide triphosphates by HPLC. Monolayers of L929 cells (6×10^6 cells/60-mm dish) were incubated at 37° with DHCaA, 3-deaza-DHCaA, DHCeA, or aristeromycin or without inhibitors. After incubation for 6 hr, the cells were washed with cold PBS(−), harvested into microcentrifuge tubes, and centrifuged at 12,000 rpm for 1 min. The cell pellet was suspended in 120 μl of cold trichloroacetic acid solution (final concentration, 0.3 M) and vortexed vigorously for 15 min at 4°. After centrifugation at 14,000 rpm for 1 min, the acid-soluble fraction was separated and treated for a few minutes at 4° with cold Freon containing 0.5 M tri-*n*-octylamine, with gentle vortexing (30). The mixture was then centrifuged at 14,000 rpm for 1 min, and the aqueous fraction was separated and injected onto an HPLC column (Partisil-10 SAX, 25 cm × 4.6 mm). Ribonucleoside 5′-triphosphates (CTP, UTP, ATP, and GTP) were separated by elution with 0.4 M $NH_4H_2PO_4$, pH 3.3, and acetonitrile (ratio of 10:1), at a flow rate of 2 ml/min, and were quantitated by measuring absorbance at 254 nm (31).

Stability of DHCaA, 3-deaza-DHCaA, aristeromycin, and Ado in murine L929 cell lysates. To prepare murine L929 cell lysates, cells were grown to confluent monolayers in 100-mm culture dishes as described above in Murine L929 cell cultures. Cells were harvested and lysates were prepared by repeated rapid freezing and thawing in cold hypotonic buffer as described above in Determination of cellular AdoHcy hydrolase activity. Cell debris and unfractured cells were removed by centrifugation, and the protein concentration in the supernatant was determined as described by Bradford (26). In a final volume of 1.5 ml, 600 μM final concentrations of compounds (DHCaA, 3-deaza-DHCaA, aristeromycin, or Ado) were added to a solution containing the cellular supernatant (final protein concentration, 1.7 mg/ml; approximately 600 μl) and 150 mM potassium phosphate, pH 7.2, containing 1 mM EDTA. The reaction mixtures were incubated at 37°, and at appropriate times aliquots (50 μl) were withdrawn and the reaction was stopped by the addition of formic acid (final concentration, 0.25 N). Levels of Ado, aristeromycin, DHCaA, and 3-deaza-DHCaA were determined by HPLC analysis as described above in Determination of AdoHcy content and AdoHcy/AdoMet ratio.

Results

4′-Modified analogs of aristeromycin effectively inhibit purified recombinant AdoHcy hydrolases. The 4′-modified analogs [DHCaA, 3-deaza-DHCaA, (4′*R*)-4′-vinyl-DHCaA, and (4′*R*)-4′-methyl-DHCaA] chosen for this study were shown to be potent inhibitors of recombinant rat liver and recombinant human placental AdoHcy hydrolases. In an earlier study these compounds were shown to be potent inhib-

TABLE 1

Inhibitory activity of 4′-modified analogs of aristeromycin and DHCeA against purified recombinant human placental or rat liver AdoHcy hydrolases and against cellular AdoHcy hydrolase in intact murine L929 cells

Purified recombinant AdoHcy hydrolase isolated from induced *E. coli* cells transformed with either the plasmid pPROKcd20 (which contains the cDNA encoding recombinant human placental AdoHcy hydrolase) or pPUCSAH (which contains the cDNA encoding recombinant rat liver AdoHcy hydrolase) was used to determine the K_i and k_2 values for the 4′-modified analogs of aristeromycin and DHCeA. The enzyme activity was measured in the synthetic direction using the HPLC method and equations described in Experimental Procedures. The ability of these analogs to inhibit the cellular enzyme in intact murine L929 cells was determined by incubating monolayers of L929 cells (1×10^7 cells/100-mm dish) with various concentrations of the inhibitors. The enzyme activity was measured in the hydrolytic direction using [2,8-^{3}H]AdoHcy as the substrate as described in Experimental Procedures. The concentration of the drug needed to inhibit AdoHcy hydrolase activity by 50% (I_{50}) was determined from a plot of the percentage of residual activity verses inhibitor concentration. Standard errors were calculated using the Cricket Graph program (version 1.3.1; Cricket Software, Malvern, PA).

Compounds	Recombinant enzymes						L929 enzyme, I_{50}[a]
	K_i		k_2		k_2/K_i		
	Human	Rat	Human	Rat	Human	Rat	
	nM		min^{-1}		$\mu M^{-1}\ min^{-1}$		nM
DHCaA	33	51	0.148	0.25	4.5	5.0	10.7 ± 2.0
3-Deaza-DHCaA	96	88	0.191	0.28	2.0	2.9	13.4 ± 2.2
(4′*R*)-4′-Vinyl-DHCaA	29	35	0.021	0.045	0.74	1.3	17.4 ± 2.3
(4′*R*)-4′-Methyl-DHCaA	37	33	0.024	0.031	0.64	0.94	36.5 ± 2.9
DHCeA	85	62	0.031	0.040	0.36	0.65	57.1 ± 2.5

[a] Values are means ± standard errors.

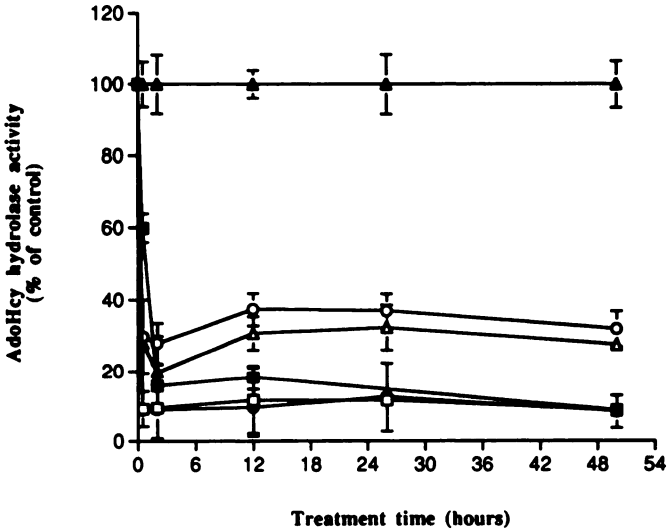


Fig. 3. Inhibition of murine L929 cell AdoHcy hydrolase by treatment with 0.1 μM concentrations of the 4′-modified analogs of aristeromycin and DHCeA. Cultures of L929 cells (1×10^7 cells/100-mm dish) were incubated with 0.1 μM DHCaA (□), 3-deaza-DHCaA (●), (4′*R*)-4′-vinyl-DHCaA (○), (4′*R*)-4′-methyl-DHCaA (△), or DHCeA (■), or were not treated (▲), for various times at 37°. The cells were harvested and lysed and the AdoHcy hydrolase activity was determined using [2,8-^{3}H]AdoHcy as the substrate, as described in Experimental Procedures. In control samples not treated with inhibitors, the AdoHcy hydrolase activity was 12.9 pmol of AdoHcy hydrolyzed/min/mg of protein. The values expressed are averages of triplicate assays.

itors of purified bovine liver AdoHcy hydrolase (17). In an effort to see whether the ability of these compunds to inhibit AdoHcy hydrolase was consistent with AdoHcy hydrolases from different sources, the recombinant enzymes were tested. The inhibitors were consistent in their ability to inhibit AdoHcy hydrolases, with K_i values less than 0.10 μM and k_2/K_i ratios of greater than 0.5 (Table 1). These values were also consistent with the ability of these compounds to inhibit purified bovine liver AdoHcy hydrolase (data not shown). The inhibitory effects of DHCeA on the purified recombinant enzymes were included as a point of reference. DHCeA had a K_i value similar to that of DHCaA or 3-deaza-DHCaA. However, in contrast to DHCaA and 3-deaza-DHCaA, DHCeA had a much smaller k_2 value, which resulted in a significantly lower k_2/K_i ratio for DHCeA (0.36 for the recombinant human placental enzyme) than for DHCaA (4.5 for the same enzyme) (Table 1). Because of their potent inhibition of AdoHcy hydrolases, these compounds were chosen for further study of their inhibitory effects on murine L929 cell AdoHcy hydrolase and L929 cell metabolism of AdoHcy and AdoMet.

4′-Modified analogs of aristeromycin effectively inhibit murine L929 cell AdoHcy hydrolase *in vivo*. Treatment of monolayers of murine L929 cells with these compounds caused a concentration-dependent inhibition of cellular AdoHcy hydrolase (data not shown). The concentrations of these compounds needed to inhibit cellular AdoHcy hydrolase by 50% (I_{50} values) were consistent with the ability of these compounds to inhibit the purified recombinant AdoHcy hydrolases, as shown in Table 1, and purified bovine liver AdoHcy hydrolase (17). DHCaA and 3-deaza-DHCaA were the most effective compounds at inhibiting L929 cell AdoHcy hydrolase, with I_{50} values of 10.7 nM and 13.4 nM, repectively, compared with an I_{50} value of 57.1 nM for DHCeA. These differences in I_{50} values were also consistent with their respective differences in k_2/K_i values (Table 1). DHCaA or 3-deaza-DHCaA (0.1 μM) was able to inhibit 90% of the AdoHcy hydrolase activity in the L929 cells within the first hour of treatment and maintained this level of inhibition for at least 48 hr (Fig. 3).

4′-Modified analogs of aristeromycin can effectively raise the intracellular levels of AdoHcy and the AdoHcy/AdoMet ratio and maintain elevated levels for at least 24 hr. Treatment of murine L929 cells with these inhibitors caused a concentration-dependent accumulation of intracellular AdoHcy (Fig. 4A). Because the concentration of intracellular AdoMet remained relatively constant (data not shown), a concentration-dependent increase in the AdoHcy/AdoMet ratio was observed (Fig. 4B). DHCaA and 3-deaza-DHCaA can be differentiated from the other inhibitors by their more potent effects in elevating AdoHcy levels and AdoHcy/AdoMet ratios. Treatment of murine L929 cells with these inhibitors (0.1 μM)

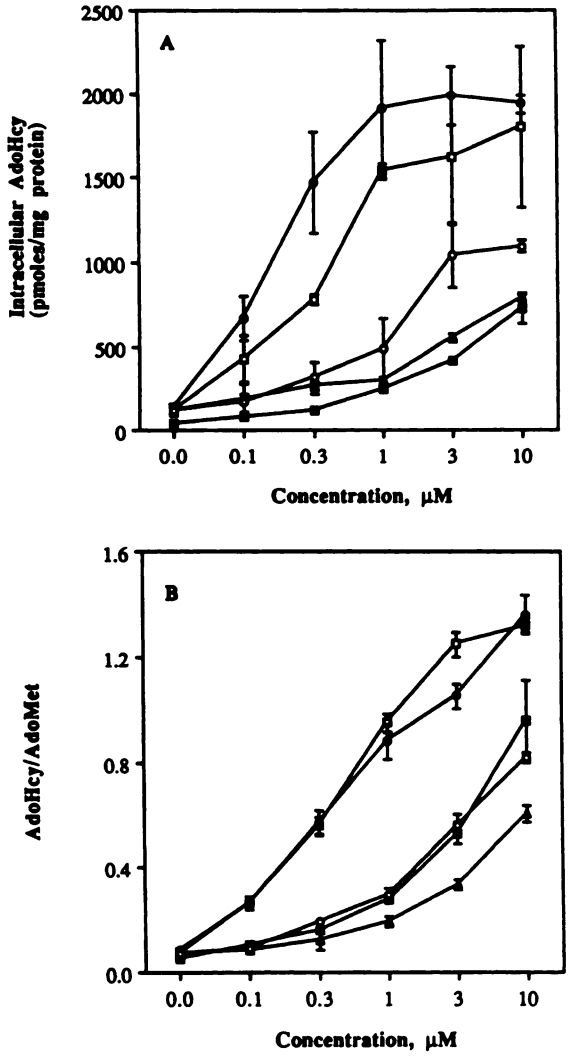


Fig. 4. Concentration-dependent elevation of intracellular AdoHcy levels (*A*) and the intracellular AdoHcy/AdoMet ratio (*B*) in murine L929 cells after exposure of the cells to 4′-modified analogs of aristeromycin or DHCeA. Monolayers of L929 cells (5×10^6 cells/60-mm dish) were treated with various concentrations of DHCaA (□), 3-deaza-DHCaA (●), (4′*R*)-4′-vinyl-DHCaA (△), (4′*R*)-4′-methyl-DHCaA (○), or DHCeA (■) for 24 hr at 37°. Cells were harvested and lysed, the intracellular levels of AdoHcy and AdoMet were determined by HPLC, and the protein concentrations were determined by staining as described in Experimental Procedures. The values expressed are averages of triplicate assays.

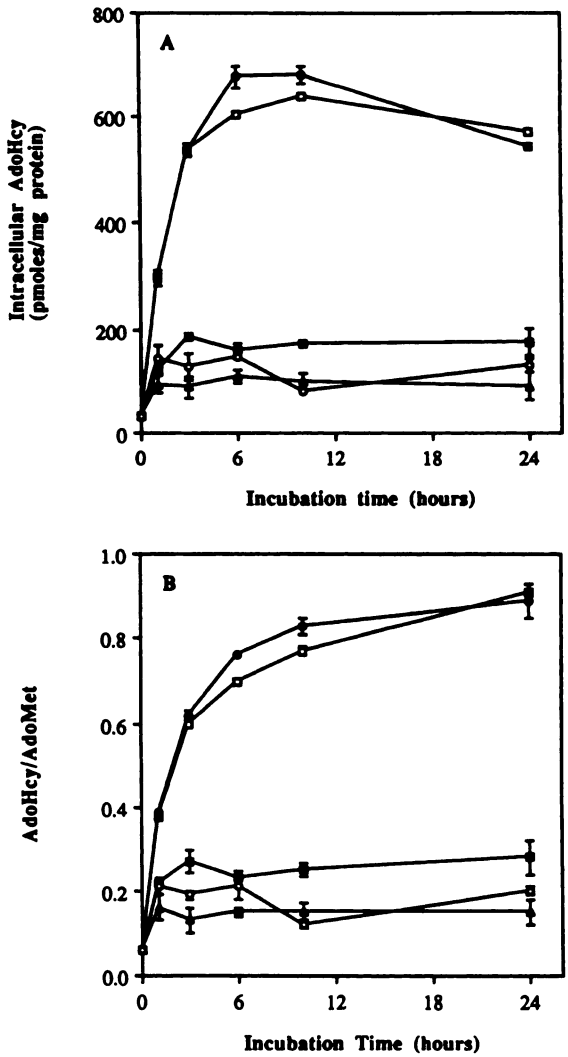


Fig. 5. Time course of the changes in the intracellular contents of AdoHcy (*A*) and the AdoHcy/AdoMet ratio (*B*) in L929 cells treated with 0.1 μM concentrations of AdoHcy hydrolase inhibitors. Monolayers of L929 cells (5×10^6 cells/60-mm dish) were treated with 0.1 μM DHCaA (□), 3-deaza-DHCaA (●), (4′*R*)-4′-vinyl-DHCaA (○), (4′*R*)-4′-methyl-DHCaA (△), or DHCeA (■) for various times. The cells were harvested and lysed and the intracellular levels of AdoHcy and AdoMet were determined by HPLC as described in Experimental Procedures. The values expressed are averages of triplicate assays.

caused a rapid increase in the intracellular concentration of AdoHcy within the first 3 hr (Fig. 5A). The intracellular levels of AdoHcy continued to increase in those cells treated with DHCaA and 3-deaza-DHCaA for an additional 3 hr, reaching a plateau level at >600 pmol of AdoHcy/mg of protein. Similar increases in AdoHcy/AdoMet ratios were also observed (Fig. 5B). During the first 6 hr of incubation the intracellular concentration of AdoMet remained relatively constant (data not shown). However, after 24 hr of incubation the intracellular levels of both AdoHcy and AdoMet decreased slightly from the values observed after 6 or 12 hr of incubation (Fig. 5). Interestingly, the ratio of AdoHcy/AdoMet at 24 hr was slightly higher than the ratios observed after 6 or 12 hr of incubation.

DHCaA, 3-deaza-DHCaA, and DHCeA are specific for AdoHcy hydrolase in murine L929 cells. These compounds were not substrates for Ado kinase, had no significant effects on the intracellular ribonucleotide pools, and showed no metabolic depletion after 6 hr of incubation with cellular lysates. Treatment of L929 cell monolayers with aristeromycin resulted in a significant decrease in the intracellular levels of ribonucleoside 5′-triphosphates and the formation of triphosphate analogs (Table 2; Fig. 6E). The formation of similar nucleotide metabolites was not detected in cell cultures treated with 10 μM DHCaA, 3-deaza-DHCaA, or DHCeA (Fig. 6, B, C, and D, respectively). In addition, these compounds did not produce any effect on the ribonucleotide pools (Table 2). Further, when aristeromycin was incubated with murine L929 cell lysates >60% of the drug was metabolized within 6 hr, whereas no reductions in the concentrations of DHCaA or 3-deaza-DHCaA were detected after incubation with cell lysates, as shown in Fig. 7.

TABLE 2

Effects of 10 μM DHCaA, 3-deaza-DHCaA, DHCeA, or aristeromycin on intracellular ribonucleoside 5′-triphosphate levels in murine L929 cells

After treatment of monolayers of L929 cells (1 × 10^7 cells/100-mm dish) with 10 μM concentrations of DHCaA, 3-deaza-DHCaA, DHCeA, or aristeromycin for 6 hr, cells were harvested, and the concentrations of ribonucleoside 5′-triphosphates were determined by HPLC analysis as described in Experimental Procedures. The values presented are averages of quadruplicate assays. The values in parentheses are percentages of control values. The formation of carbocyclic ATP (C-ATP) and carbocyclic GTP (C-GTP) was detected in cells treated with aristeromycin.

Compounds	Ribonucleoside 5′-triphosphates					
	CTP	UTP	ATP	C-ATP	GTP	C-GTP
	pmol/10^6 cells					
Control (0 μM)	452 ± 7 (100%)	793 ± 15 (100%)	2829 ± 87 (100%)		583 ± 42 (100%)	
DHCaA	479 ± 68 (106%)	792 ± 101 (100%)	3179 ± 297 (112%)		619 ± 47 (106%)	
3-Deaza-DHCaA	466 ± 15 (103%)	769 ± 45 (97%)	3062 ± 83 (108%)		592 ± 29 (102%)	
DHCeA	455 ± 32 (101%)	823 ± 58 (104%)	3125 ± 128 (110%)		641 ± 59 (110%)	
Aristeromycin	269 ± 11 (60%)	729 ± 55 (92%)	2277 ± 117 (80%)	695 ± 51	348 ± 54 (60%)	408 ± 11

Discussion

A good correlation exists between the antiviral effectiveness of Ado analogs and their ability to inhibit the cellular enzyme AdoHcy hydrolase (13). AdoHcy hydrolase inhibitors are also potent as broad-spectrum antiviral agents, inhibiting the replication of a variety of (−)-strand RNA viruses (including such clinically important viruses as measles, respiratory syncyntial virus, influenza A and B, and rabies) and double-stranded RNA viruses (including rotavirus, a major cause of gastroenteritis in children) (9). They are not particularly active against (+)-strand RNA viruses or DNA viruses (except for vaccinia and African swine fever viruses).

AdoHcy hydrolase provides the only known mechanism for AdoHcy catabolism in eukaryotes (1), catalyzing its hydrolysis to Ado and Hcy as shown in Fig. 1. Inhibition of AdoHcy hydrolase results in increased cellular levels of AdoHcy and an increased ratio of AdoHcy/AdoMet. The increased cellular AdoHcy levels that result from AdoHcy hydrolase inhibition have been correlated with the antiviral activity of these inhibitors (13). Undermethylation at the 5′ terminus of viral mRNA induced by inhibition of cellular AdoHcy hydrolase has also been correlated with inhibition of viral replication (32).

Aristeromycin was shown earlier to be a potent inhibitor of AdoHcy hydrolase *in vitro* (14) and an effective antiviral agent (15). However, in cell culture systems aristeromycin is a poor inhibitor of AdoHcy hydrolase because it is rapidly metabolized by Ado deaminase and Ado kinase (24). High levels of toxicity are also associated with aristeromycin, precluding its usefulness as a clinical antiviral agent. The toxicity associated with aristeromycin is thought to occur through its formation of nucleotide metabolites (15, 23). Aristeromycin 5′-monophosphate serves as a substrate for AMP deaminase, converting it to the IMP analog of aristeromycin (23, 33), which is then converted to phosphates of carbocyclic guanosine. Complete blockage of the utilization of hypoxanthine and guanine upon treatment of cells with aristeromycin is explained by the ability of carbocyclic GMP to serve as a good inhibitor of hypoxanthine phosphoribosyltransferase (15, 23).

Neplanocin A is another nucleoside analog inhibitor of AdoHcy hydrolase (4). Neplanocin A inhibits AdoHcy hydrolase by a "cofactor-depletion" mechanism involving the reduction of enzymatically bound NAD^+ to NADH with the simultaneous oxidation of the substrate to form a 3′-keto nucleoside (34). Because removal of the 5′-hydroxymethyl group would clearly preclude 5′-phosphorylation by Ado kinase and because it was shown that adenine nucleosides that did not contain a free 5′-hydroxymethyl group were poor substrates for Ado deaminase (35), a series of 4′-modified analogs of neplanocin A were synthesized (19). These compounds were shown to retain their inhibitory effects on AdoHcy hydrolase (20), to retain their ability to raise the intracellular AdoHcy/AdoMet ratio (18), and to effectively inhibit vaccinia virus replication in murine L929 cells (21). These compounds were also shown to have dramatically reduced cytotoxicity and improved stability when incubated with L929 cell lysates (18). The most effective compound in this series of inhibitors is DHCeA. Given the success of the 4′-modified analogs of neplanocin A in reducing cytotoxicity while retaining inhibitory properties towards AdoHcy hydrolase, a series of 4′-modified analogs of aristeromycin were synthesized and shown to be potent inhibitors of purified bovine liver AdoHcy hydrolase (17). DHCeA, therefore, serves as a good reference or benchmark compound with which to compare the effectiveness of the 4′-modified analogs of aristeromycin.

In this study the 4′-modified analogs of aristeromycin were shown to be potent inhibitors of purified recombinant rat liver and recombinant human placental AdoHcy hydrolase (Table 1). A strong correlation exists between the abilities of these compounds to inhibit the purified enzymes and to inhibit the cellular AdoHcy hydrolase in intact murine L929 cells, as seen by comparing the K_i values with the IC_{50} values for these compounds listed in Table 1. Clearly, DHCaA and 3-deaza-DHCaA are more effective inhibitors of AdoHcy hydrolase than is DHCeA. As in previous studies with DHCeA (18), DHCaA and 3-deaza-DHCaA were shown in this study to effectively inhibit cellular AdoHcy hydrolase in both a time- and concentration-dependent manner (Fig. 3; Table 1).

In previous studies DHCeA was shown to increase the intracellular levels of AdoHcy and the AdoHcy/AdoMet ratio (18). The intracellular levels of AdoHcy and the AdoHcy/AdoMet ratio increased over the first 6 hr of exposure to DHCeA and then remained at high levels for at least 72 hr (18). As shown in Figs. 4 and 5, DHCaA and 3-deaza-DHCaA are much more effective than is DHCeA at raising intracellular levels of AdoHcy and the AdoHcy/AdoMet ratio and maintaining these high levels. This sustained effect on cellular metabolism can be explained by the cellular stability of these compounds (Fig. 7), in combination with the slow turnover of cellular AdoHcy hydrolase (3).

It is interesting to note that at 100 nM concentrations DHCeA, DHCaA, and 3-deaza-DHCaA all produce approximately equal levels of inhibition (approximately 90%) of L929

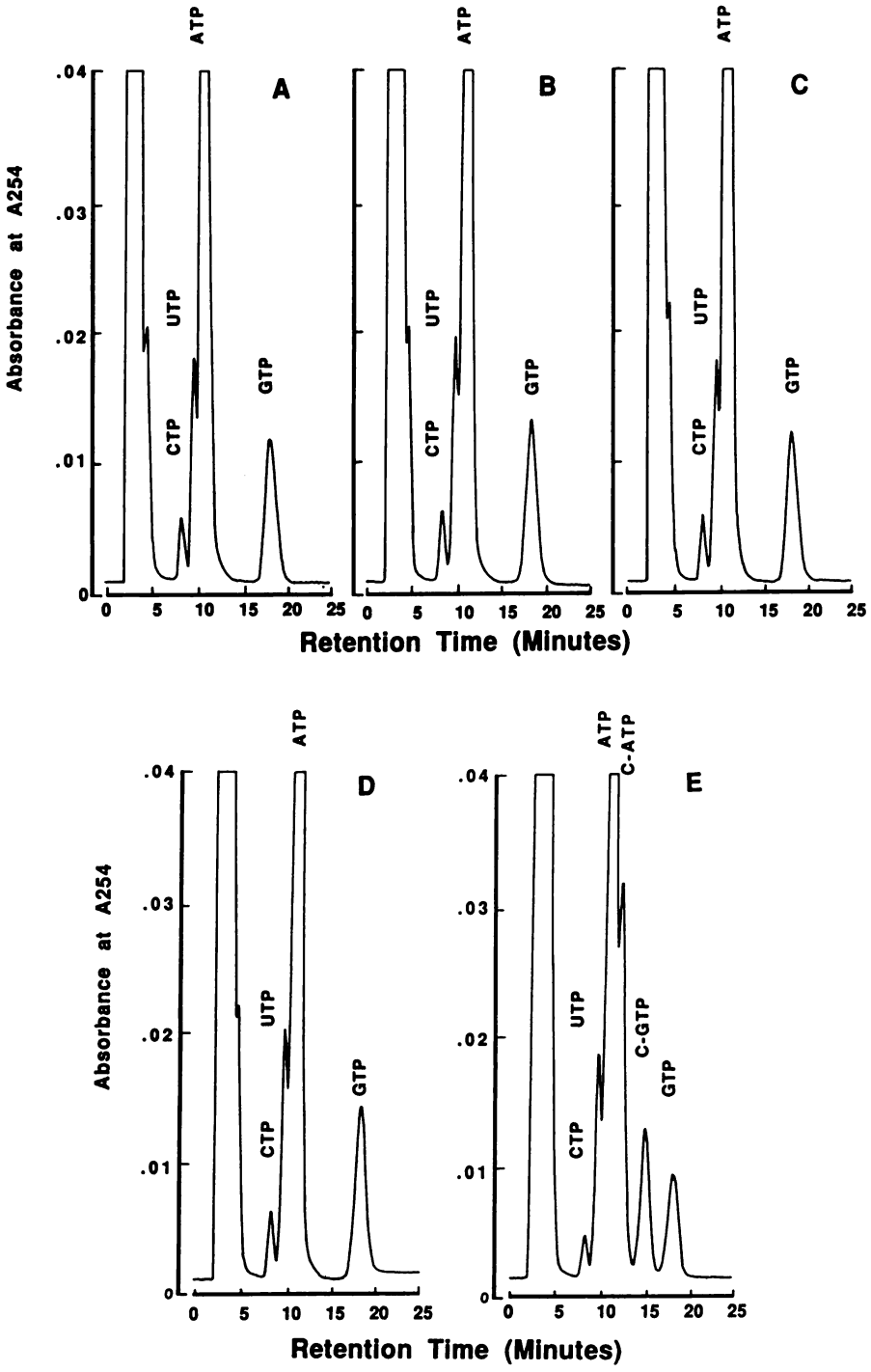


Fig. 6. HPLC chromatograms of ribonucleoside 5′-triphosphates from L929 cells treated with DHCaA (B), 3-deaza-DHCaA (C), DHCeA (D), or aristeromycin (E) or not treated with inhibitors (A). Monolayers of L929 cells (approximately 5 × 10^6 cells/60-mm dish) were treated with 10 μM concentrations of AdoHcy hydrolase inhibitors for 6 hr at 37°. The cells were harvested and lysed and the intracellular levels of ribonucleoside 5′-triphosphates were determined as described in Experimental Procedures. The formation of carbocyclic ATP (*C-ATP*) and carbocyclic GTP (*C-GTP*) was detected in the cells treated with aristeromycin (E).

cellular AdoHcy hydrolase (Fig. 3). However, at the same concentration their effects on intracellular AdoHcy levels and AdoHcy/AdoMet ratios (Fig. 5) are quite different. For example, at a 100 nM concentration DHCeA elevates the AdoHcy/AdoMet ratio to approximately 0.2 at 24 hr, whereas DHCaA and 3-deaza-DHCaA elevate the AdoHcy/AdoMet ratio to approximately 0.9 (Fig. 5). These effects on AdoHcy/AdoMet ratios would not have been predicted, based on the inhibition data shown in Fig. 3. This apparent discrepancy between the AdoHcy hydrolase inhibitory activity and the effects of inhibitors on AdoHcy/AdoMet ratios may be related to the observation that even the most potent inhibitors of this enzyme do not totally inhibit AdoHcy hydrolase in cell culture (3, 29). Even in this study, we observed that cells treated with 100 nM concentrations of DHCeA, DHCaA, or 3-deaza-DHCaA retained approximately 10–15% residual AdoHcy hydrolase activity (Fig. 3). Earlier, our laboratory proposed (29) that there exist inhibitor-sensitive and inhibitor-insensitive isoforms of AdoHcy hydrolase in L929 cells. In fact, two isoforms of this enzyme, one inhibitor sensitive and the other inhibitor insensitive, have been separated from homogenates of L929 cells by chromatographic means (36). In addition, we have observed that this inhibitor-insensitive form of AdoHcy hydrolase appears to contribute to the actual synthesis of AdoHcy from Ado and Hcy (29). Thus, although all of the inhibitors used in this study have significantly different effects on the inhibitor-sen-

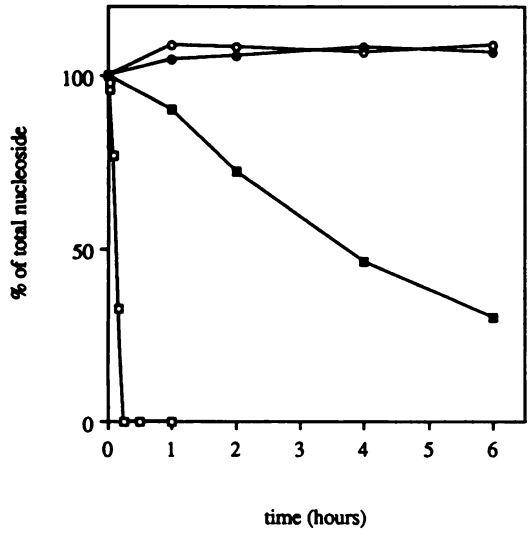


Fig. 7. Stability of DHCaA, 3-deaza-DHCaA, aristeromycin, and Ado in murine L929 cell lysates. Cell lysates were prepared with cold hypotonic buffer by the method described in Experimental Procedures. The incubation mixture contained 150 mM potassium phosphate, pH 7.2, 1.0 mM EDTA, 600 μM DHCaA (○), 3-deaza-DHCaA (●), aristeromycin (■), or Ado (□), and cell lysate (1.7 mg of protein/ml), in a total volume of 1.5 ml. The reaction mixtures were incubated at 37°, and at appropriate times aliquots (50 μl) were withdrawn and the reaction was stopped by the addition of formic acid (final concentration, 0.25 N). Levels of DHCaA, 3-deaza-DHCaA, aristeromycin, and Ado were determined by HPLC analysis as described in Experimental Procedures.

sitive forms of the enzyme, the differences observed in their abilities to alter intracellular levels of AdoHcy are even more dramatic and may relate to more subtle effects on the inhibitor-insensitive form of the enzyme that are not measurable by existing methodology.

DHCaA, 3-deaza-DHCaA, and DHCeA showed little effect on the intracellular nucleoside 5′-triphosphate pools (Table 2). In addition, analog triphosphates were not detected, as shown in Fig. 6 (B, C, and D, for DHCaA, 3-deaza-DHCaA, and DHCeA, respectively). However, triphosphate analogs of aristeromycin were detected (Fig. 6E). The formation of these metabolites of aristeromycin was consistent with earlier reports describing their formation (23, 33). The inability of DHCaA and 3-deaza-DHCaA to form triphosphate metabolites is also consistent with their stability when incubated with L929 cell lysates (Fig. 7), whereas the instability of aristeromycin in L929 cell lysates (Fig. 7) is also consistent with the formation of aristeromycin nucleoside triphosphate metabolites shown in Fig. 6E. It should be noted that DHCeA, an analog of neplanocin A, showed similar stability characteristics in L929 cell lysates (18).

In a separate study, DHCaA and 3-deaza-DHCaA were found to be potent inhibitors of vaccinia virus replication in murine L929 cells (IC_{50} values of 0.17 μM and 0.13 μM, respectively) and to have greatly reduced cytotoxicity (ID_{50} values of 73 μM and 27.4 μM, respectively) (37). In this same study, aristeromycin was shown to be a weak inhibitor of vaccinia virus replication (IC_{50} value of 6.62 μM) and to produce cytocidal effects (ID_{50} value of 4.3 μM) on murine L929 cells. In contrast, DHCaA and 3-deaza-DHCaA were shown to produce cytostatic effects on cell growth, rather than cytocidal effects (37). It should also be noted that the antiviral effects of DHCaA and 3-deaza-DHCaA were correlated with their abilities to elevate intracellular ratios of AdoHcy/AdoMet (37). These results confirm that the antiviral effects of aristeromycin are mediated through its inhibition of AdoHcy hydrolase and that transformation of aristeromycin by cellular Ado kinase mediates its cytocidal properties. The antiviral activities of these compunds against a broad spectrum of viruses and the *in vivo* efficacy of these compounds are currently being evaluated. Preliminary results suggest that DHCaA and 3-deaza-DHCaA are broad-spectrum antiviral agents, being particularly effective against vesicular stomatitis virus, vaccinia virus, parainfluenza virus, reovirus, and rotavirus.[1] This spectrum of antiviral activity is characteristic of AdoHcy hydrolase inhibitors (9, 16, 22).

Finally, because these compounds have the ability to act as specific inhibitors of AdoHcy hydrolase, they can be used as biochemical tools to further elucidate the metabolic role(s) of AdoHcy hydrolase in both normal and virus-infected cells. Information gained about these processes will undoubtedly be useful in the design of even more effective and less toxic broad-spectrum antiviral agents.

Acknowledgments

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Send reprint requests to: Dr. Ronald T. Borchardt, Department of Pharmaceutical Chemistry, Room 3006, Malott Hall, The University of Kansas, Lawrence, KS 66045.